Identification of UK populations and determination of pupal age of the forensically important blowfly Calliphora vicina and Calliphora vomitoria

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IDENTIFICATION OF UK POPULATIONS AND DETERMINATION OF PUPAL AGE OF THE BLOWFLY FORENSICALLY IMPORTANT *CALLIPHORA* ~~SPECIES~~ VICINA AND CALLIPHORA VOMITORIA

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Abstract

Forensic entomologists most commonly utilise insects for the estimation of time since death of a discovered corpse. This work addressed two of the challenges encountered when providing estimates of minimum time since death.

Entomological techniques depend upon accurate identification of insect species. The first aim of this work was to use DNA molecular markers for differentiation of forensically important *Calliphora* species and populations within the UK. Regions of both the mitochondrial and nuclear genome were examined for potential markers. The mitochondrial cytochrome oxidase subunit I was found to distinguish between *Calliphora vicina* and *Calliphora vomitoria*. This region contained low intraspecific variation and therefore provided an efficient interspecific marker for these species. The nuclear xanthine dehydrogenase gene was found to contain enough intraspecific variation to group populations of *C. vicina* and *C. vomitoria* and indicate that a definite genetic structure exists in English *Calliphora* populations.

The work also examined the structure of the mitochondrial control region, which had not been examined previously in these species.

The developmental timings of two *C. vicina* populations were also assessed and significant statistical differences were found between the duration three out of five immature stages for West and East England populations at a constant temperature.

To establish time since death, an entomologist requires accurate assessment of the age of insects discovered associated with a corpse. The other aim of this work was to use molecular techniques to determine the age of immature forms of forensically important fly species. Throughout the developmental lifecycle of insects different genes will be expressed at specific time points. These temporally expressed genes could provide markers as to the age of an insect. This work located several markers whose expression was upregulated at specific times during the *C. vicina* pupal stage.

Table of Contents

List of Tables

List of Figures

List of Appendices

Abbreviations

ADD/ADH	Accumulated degree day/hour
Amino Acids	see table for three and one letter codes
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
COI	cytochrome oxidase subunit I
COOH	Protein carboxyl terminal
ddH_2O	distilled deionised water
DEG	Differentially expressed gene
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
kb	kilobase
L1 L2 L3	Larval instar 1,2,3
MGB	minor groove binder
MHC	Myosin heavy chain
mtDNA	mitochondrial DNA
NH_2	Protein amino terminal
nuDNA	nuclear DNA
OS	Ordnance Survey
PCR	Polymerase Chain Reaction
PMI	Post-Mortem Interval
PSA	prostate specific antigen
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
STR	Short tandem repeat
TAE	Tris-acetic acid-EDTA
tRNA	Transfer RNA
UV	ultraviolet
XDH	xanthine dehydrogenase

Abbreviations/cont.

Amino Acids

A	**Ala**	Alanine
C	**Cys**	Cysteine
D	**Asp**	Aspartic Acid
E	**Glu**	Glutamic Acid
F	**Phe**	Phenylalanine
G	**Gly**	Glycine
H	**His**	Histidine
I	**Ile**	Isoleucine
K	**Lys**	Lysine
L	**Leu**	Leucine
M	**Met**	Methionine
N	**Asn**	Asparagine
P	**Pro**	Proline
Q	**Gln**	Glutamine
R	**Arg**	Arginine
S	**Ser**	Serine
T	**Thr**	Threonine
V	**Val**	Valine
W	**Trp**	Tryptophan
Y	**Tyr**	Tyrosine

Nucleotide Bases

A	Adenine
C	Cytosine
G	Guanine
T/U	Thymine/Uracil
M	A or C
R	A or G
W	A or T
S	C or G
Y	C or T
K	G or T
V	A or C or G
H	A or C or T
D	A or G or T
B	C or G or T
N	A or C or G or T
-	not A, C, G, T

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Chapter 1

The role of Forensic Entomology in Forensic Science

In cases of unexplained death any arthropods, especially insects, associated with a corpse will provide an investigator with evidence or intelligence. The concept of applying the study of insects to the investigation of crime is known as forensic entomology and has been widely used since the early 1900s (Benecke 2001). Insects are most commonly used by forensic investigators to estimate the time of death or post-mortem interval (PMI) of human corpses.

1.1 Post Mortem Interval

According to Sperling *et al.* (1994), insects allow for more precise estimates of the minimum PMI than are possible by any other means when death has occurred three days or more before discovery. After 72h traditional medical observations (e.g. *algor mortis, rigor mortis* and *livor mortis*) are no longer evident (Anderson 2004). Similarly, these post-mortem conditions will be absent in burnt or partial remains of bodies and therefore in these cases insect evidence will be very important.

The minimum PMI can be estimated in the following three ways.

1.1.1 Accumulated Degree Day/Hour Model

Insects are poikilothermic (cold-blooded). Their body temperature is influenced directly by the temperature of their microenvironment. There is a direct relationship between the temperatures experienced by an insect and its developmental rate. At low temperatures the relationship between developmental rate and temperature is non-linear. As temperature increases, developmental rate becomes proportional to temperature. Developmental rate plateaus as the optimum temperature is reached and decreases rapidly after this.

Even though it was recognised in the early 1700s that insect developmental time is dependent on temperature (Reamur 1735, cited in Smith 1986), it wasn't until the mid 1800s that methods were established to exploit this relationship and use temperature to predict insect development (Pradhan 1946, Candolle 1855 cited in Sharpe and DeMichele 1977). The model most commonly utilised by entomologists from many

disciplines, including forensic science, focuses upon the temperature range where the relationship of development rate and temperature is constant. This model is known as the degree-day or degree-hour model and was introduced by Candolle (1855 cited in Sharpe and DeMichele 1977). The model assumes that no development occurs below a designated minimum developmental threshold. The number of degree-days (or hours) is the product of the temperature above the minimum developmental threshold and the time spent at that temperature. Insects develop through distinct identifiable stages. An accumulated number of degree-days (ADD) or degree-hours (ADH) are associated with specific development stages for each insect species. This value is usually derived empirically for each species. A combination of the developmental stage present on a corpse, the ADD necessary to reach this stage for the particular species and the previous temperature conditions will disclose the time when eggs were laid and thus the minimum PMI.

Forensic entomologists use corrected temperatures from local weather stations after establishing how temperatures in the vicinity of the corpse after discovery compare to the weather station data (Archer 2004).

1.1.2 Isomegalen-diagrams

A method that allows an entomologist to estimate the age of an insect and thus minimum PMI is to use an empirically derived isomegalen-diagram. This consists of several plots of time since oviposition (egg laying) against constant temperature for several larval lengths (Reiter 1984; Grassberger and Reiter 2001). More recently, Day and Wallman reported that the width of a larva could also indicate age (Day and Wallman *in press*).

The temperature dependent models produce good estimates for low PMI. When time since death is longer another method using entomological evidence is used.

1.1.3 Faunal succession and carrion decomposition

Mégnin (1894 cited in Grassberger and Reiter 2001), illustrated that there was a succession of insect species on human cadavers. Initially species invade carrion to feed and oviposit and other invertebrates arrive to predate on these insects. He identified eight stages of decomposition and the species associated with each. These were later reclassified into the following five stages of decomposition. Fresh, bloated,

active decay, post decay and dry decay stages (Bornemissza 1957). Decomposition will always contain these stages although the duration of each depends on the environmental conditions (e.g. temperature, humidity or presence of carnivorous animals) and the state of the body (e.g. external wounds allow quick invasion of insects and external bacteria, Campobasso *et al.* 2001). It is the faunal succession associated with these stages that provides forensic entomologists with another method for estimating the PMI. The presence or absence of a species can indicate the stage of decomposition of a corpse and provide an estimate of time since death.

Whilst the exact species present will depend upon geographic region the general pattern identified by Bornemissza (1957) begins with Calliphoridae larvae (blowfly; Diptera) feeding on the putrefying corpse. After the appearance of dipteran species on the corpse, predators such as Staphylinidae (rove beetles) and Histeridae (histerid beetles) arrive to feed on blowfly larvae. Blowfly pupae are also parasitised by the immature forms of the wasp *Nasonia vitripennis* (Walker). This wasp oviposits directly into the pupal case approximately 24h into the pupal phase (Grassberger and Frank 2003). Their larvae feed upon the developing fly eventually killing it. Once fully developed the wasp larvae pupate and then emerge as adults, which chew holes in the blowfly puparium and leave. The wasp mainly parasitises *Chrysomya rufifacies* (Macquart) and *Chrysomya albiceps* (Wiedemann) as their wandering larvae do not bury into the soil unlike *Calliphora* species and are thus more exposed during the pupal stage. The lifecycle of parasites can also be used to establish minimum PMI.
As decomposition of the carcass continues, Piophilidae flies (cheese skippers) arrive. Once the corpse has skeletonised the final wave of insects appears including Dermestidae (skin beetles), Cleridae (bone beetles) along with clothes moth larvae.

1.2 Calliphoridae (Diptera)

Calliphoridae (blowflies) are considered the most important family forensically as they are normally the first invaders of surface carrion (Bornemissza 1957). Calliphorid species therefore provide information for estimating minimum PMI using the ADD model of isomegalen-diagrams (Sections 1.1.1 and 1.1.2). Calliphorid immature stages require a protein rich diet for development and the family can be divided according to their larval feeding habits. Saprophagous larvae, *Calliphora*

species (bluebottles), live in decaying vertebrate matter; obligate ectoparasites. *Chrysomya beziana* and *Cochliomyia hominivorax* species (screwworm flies), feed on living animal tissue and facultative ectoparasites, *Lucilia* species (greenbottles), live as both sarcophages and ectoparasites. In Europe, *Calliphora* usually arrive at carrion first whereas in the USA, *Calliphora* arrive after *Lucilia* and *Cochliomyia* (Smith 1986). As this work is based upon UK species and populations, *Calliphora* species will be focused upon.

1.2.1 *Calliphora* species

Calliphora are large blue flies (6-14mm) that are widely distributed around the world. The genus is especially prevalent in the Holarctic and Australian regions.

Two species of *Calliphora* are common in the UK. *Calliphora vicina* Robineau – Desvoidy (= *Calliphora erythrocephala* Macquart) is a very common species and whilst it can be found in rural areas it is especially dominant in urban areas due to its close associations with man. It is therefore the species most likely to be found on fresh corpses in lowland urban locations. Smith and Wall (1997a) found that of 241 carcasses infested with fly larvae from fields and hedgerows in England, *C. vicina* was the most prevalent species, emerging from 67% of carcasses, with a greater number from hedgerow carcasses than those out in the open. It is usually the most prevalent species on indoor corpses.

Calliphora vomitoria (Linnaeus) is the other common UK *Calliphora* species but is more rural in distribution than *C. vicina* and therefore less abundant in urban situations. The relative abundance of these two species is reversed in upland regions compared with lowland urban areas (Davies 1990), although it can be found in high numbers in areas of grassland or woodland within cities (Hwang 2004). In the Smith and Wall study (1997a), *C. vomitoria* was located on only two out of 241 carcasses but this may have been due to the corpses being mice and quail, perhaps too small to attract gravid *C. vomitoria* females. *Calliphora vomitoria* tends to oviposit on larger carcasses than *C. vicina*.

Calliphora vomitoria is often used by anglers as coarse fishing bait and consequently can be transported to areas outside its natural distribution.

Both *C. vicina* and *C. vomitoria* are cold adapted (Greenberg 1985 in Greenberg 1991). They have a higher metabolic rate than warm-adapted species at the same temperatures and are usually present most of the year (in the UK). Only in cold

winters does *Calliphora* activity cease, although they have been noted to fly on the occasional sunny day in winter (London, UK. Adams and Hall 2003).

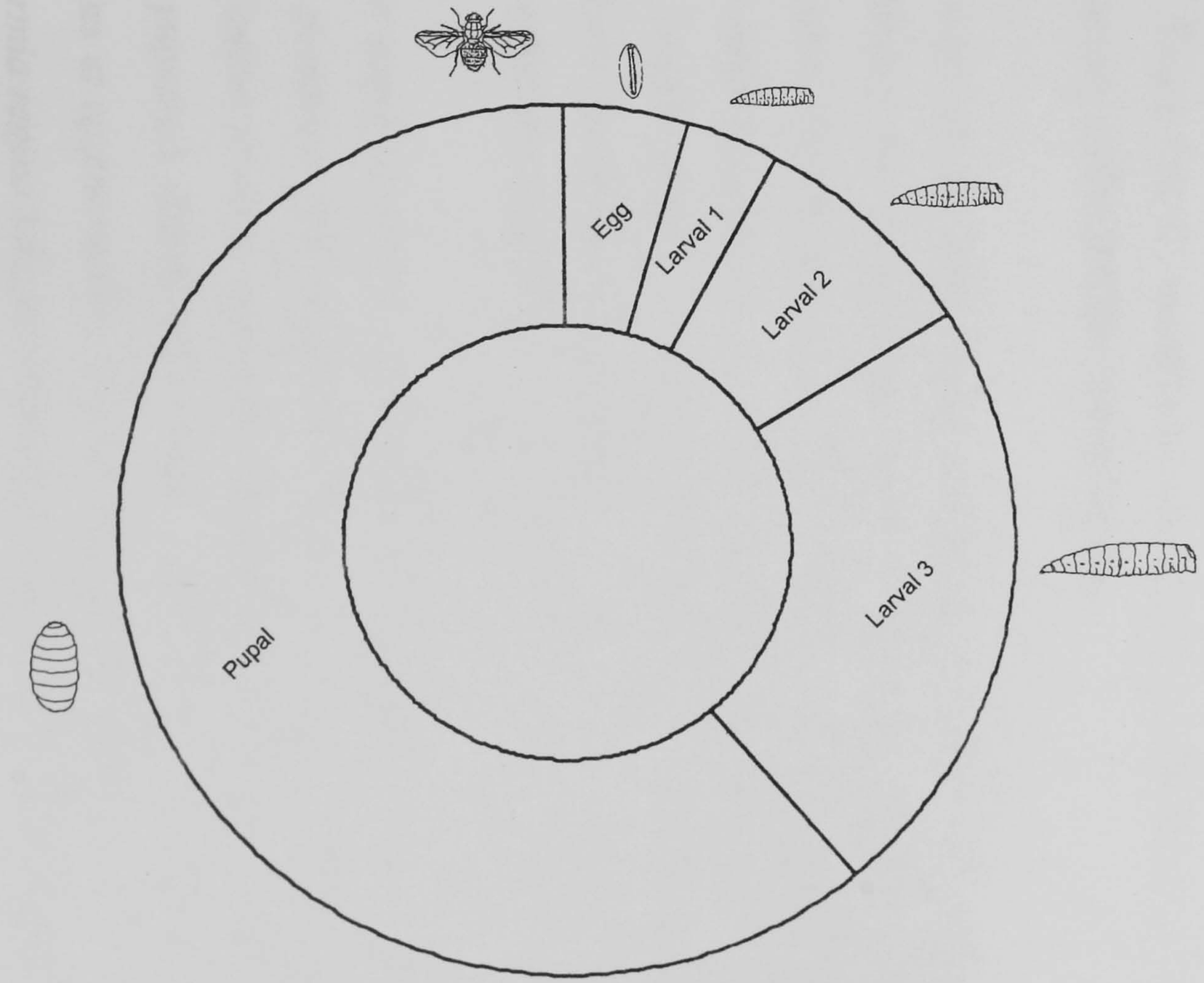
Other *Calliphora* species found in the UK include *Calliphora alpina* (Zetterstedt), *Calliphora subalpina* (Ringdahl) and *Calliphora loewi* Enderlein. These species are found in rural upland areas. Davies (1990) found that *C. alpina* was absent from the Pennines and upland Wales where *C. subalpina* is more prevalent.

The differing habitats of these UK *Calliphora* species indicate why *C. vicina* and *C. vomitoria* are the most important *Calliphora* species in forensic investigations as they inhibit areas alongside man (*C. vicina* in urban environments and *C. vomitoria* in grassland or woodland within urban areas).

1.2.2 *Calliphora* Life cycle

Calliphora species are predominantly active during daylight and therefore oviposition normally occurs on a corpse during the day. Lack of nocturnal oviposition in some regions may be due to the drop in environmental temperature overnight. Adult flies will not normally oviposit in cold periods (Smith 1986). Some calliphorid species have also been observed to oviposit in the dark. Singh and Bharti (2001) conducted night oviposition experiments outdoors in India with a minimum temperature of 16°C. Observations of night time oviposition within the laboratory (personal observation) are at temperatures higher than that of the outdoors. .

Oviposition on carrion often occurs within a few hours of death (under the right conditions of temperature and location, this can happen immediately) as female blowflies have excellent olfactory senses. Blowfly females have synchronised development of ovarioles and consequently lay batches of up to 300 eggs targeted in natural or unnatural orifices (i.e. wounds, Clery 2001). When the eggs hatch, the larvae (maggots) penetrate the flesh to feed, using protein and lipids in the carrion as an energy source. Adult flies feed on sugars (usually from nectar) for energy. Females also require protein for egg production. Duve *et al.* (1992) showed that *C.vomitoria* females fed on sugar and water showed restricted egg development and low levels of the *C. vomitoria* juvenile hormone – a compound linked to vitellogenesis (yolk formation in the developing egg).



Mean Duration of Stage	*C.vicina* h ± s.d. (% of total)	*C.vomitoria* h ± s.d. (% of total)
Egg	24.7 ± 1.3 (4.6)	23.6 ± 1.2 (4.1)
Larval 1	17.9 ± 4.9 (3.3)	15.5 ± 4.2 (2.7)
Larval 2	44.1 ± 5.7 (8.2)	47.2 ± 5.8 (8.2)
Larval 3	123.4 ± 12.9 (22.9)	195.5 ± 13.1 (34.1)
Pupal	328.5 ± 12.5 (61.0)	290.7 ± 18.1 (50.8)

Figure 1.1. Lifecycle diagram of *C. vicina* and *C. vomitoria*. Figures in table from Ames and Turner (2003). Mean duration of stages in hours for insects kept at 20°C. Diagrams of life stages drawn from figures in Smith (1989) and Erzinçlioğlu (1996).

After hatching, the immatures develop through three larval stages (see Figure 1.1). Larvae consist of twelve segments (one head, three thoracic and eight abdominal segments). Each stage increases in size and moults to form the next stage. Whilst in the third larval stage the maggots are at their largest and feed intensely on the food source. After feeding ceases, the crop (food storage organ) evacuates and the larvae migrate away from the food source, burying down into the soil, if present. This stage is known as the post-feeding or 'wandering' stage and the burying is thought to evade predators. *Calliphora vicina* larvae have been observed to travel up to 11m, the distance travelled depending on soil type (Amoret Brandt, personal communication). Gomes and Zuben (2005) showed that *C. albiceps* in the laboratory travelled a maximum of 11cm but this was conducted in containers with a maximum diameter of 50cm – the larvae may have been too confined by these dimensions and this probably does not indicate maximum distance. Greenberg (reviewed in Greenberg, 1991) carried out a series of laboratory experiments and showed that *C. vicina* larvae dispersed 3-8.1m from the food source and 50% of *Cochliomyia macellaria* Fabricius travelled 2.1m. Tessmer and Meek (1996) conducted a similar experiment in the field and found that *C. macellaria* larvae did not travel as far in the field with 59% remaining within 0-0.9m of food source.

Once buried, the larva begins to contract and the cuticle (called the puparium) begins to harden and darken from white to dark red. During the first 12h after white puparium formation the last larval moult occurs (larval-adult apolysis, Hinton 1973). The developing insect can now be referred to as a pupa and the remainder of this stage the 'pupal' stage. The phase between white puparium formation and larval-adult apolysis is termed the prepupal stage, with the developing insect, the prepupa (Fraenkel and Bhaskaran 1973; Hinton 1973; Wigglesworth 1973).

After pupal formation, the head, thorax and abdomen start to become evident within the puparium and respiratory horns are pushed through the puparium to allow respiration to occur during the pupal phase. These are situated on the 5th segment of the puparium (Erzinçlioğlu 1985). This is the last external structural change and occurs at approximately 24-26h after pupal formation at 22°C for the black blowfly *Phormia regina* Meigen (Greenberg 1991). The pupal stage is the longest of the pre-

adult stages (61% of total time until adult emergence for *C. vicina* and 50.8% for *C. vomitoria*, Figure 1.1). After 449 ADD for *C. vicina* and 727 ADD for *C. vomitoria* (Ames and Turner 2003) the adult flies emerge. The cuticle requires time to harden and the wings need to expand before the normal adult size and colour is attained. Hayes *et al.* (1999), whilst examining the greenbottle *Lucilia sericata* (Meigen), noted it took 30 ADD post-emergence for females to begin to move towards a protein source. The value for *Calliphora* females is likely to be of similar magnitude. Mating lasts for 10-12 min in *Lucilia cuprina* Robineau-Desvoidy and females store sperm, thus rarely mating more than once (Smith *et al.* 1988). Female *Calliphora* normally oviposit 4-5 days after emergence, if a suitable protein source is available (Green 1951). In the laboratory, *C. vicina* and *C. vomitoria* adults live for approximately three weeks, although they have been observed to live 100.2 days ± 3.1 (*C. vicina* males, Tribe and Webb 1979). Smith and Wall (1998) quote an average of 3-6 days (maximum of 30 days) for wild *L. sericata* adult survival. There will be several *Calliphora* generations a year although during winter the general numbers decrease (MacLeod and Donnelly 1958). Abou Zied *et al.* (2003) indicated that, in the case of *L. cuprina,* adult females lived for an average of ten days longer than males (in laboratory conditions).

Calliphora vicina has the ability to go through its lifecycle with or without a period of diapause. Diapause is the state in which insects often overwinter. The period of diapause is associated with a fall in metabolic rate, along with glycerol accumulation for cold tolerance and other species specific events. Diapause in this species is defined as a lack of pupariation after thirty days (Saunders 1987).

If *C. vicina* females are exposed to short day periods the next generation will experience diapause during the third larval stage (Vinogradova and Zinovjeva 1972). Diapause is only fully expressed if the third larval stage experiences temperatures lower than around 15°C (Vaz Nunes and Saunders 1989). If third stage larvae, even at temperatures below 15°C, experience overcrowding diapause can be side-stepped (Saunders 1997). McWatters and Saunders (1996) showed using genetic crosses of *C.vicina* strains from the North and South of England, that diapause was not only dependent upon conditions experienced but also that some strains of *C. vicina* were more predisposed to diapause than others. These researchers highlighted that whilst diapause incidence depended on maternal influence the length of diapause was a

reflection of the genetics of both parents. In England, *C. vicina* located in the North are more likely to diapause than those in the South.

Block *et al.* (1990) postulated that both *C. vicina* and *C. vomitoria* in the south probably sheltered over winter to avoid freezing thus making diapause unnecessary.

1.3 Other uses for insect evidence

Invertebrates can aid an investigator in a variety of other ways besides PMI estimation. For instance, as insect species frequently have distinct geographical distributions, their presence on a corpse outside of their normal habitat can indicate post-mortem movement of a body. Some insects are prevalent at certain seasons and therefore may indicate the time of year a crime occurred. The presence of drugs or poisons within feeding insects may give an indication of the cause of death of a corpse (Campobasso and Intron 2001). This is especially important when conventional toxicological samples are not available. Insect immature stages and pupal cases remain unchanged in the environment after body fluids and tissue have decomposed (Bourel *et al.* 2001). Pupal cases can remain for years after death (Erzinçlioğlu 1996). Drugs such as codeine, heroin, morphine, cocaine, amphetamines, antidepressants, paracetamol, barbiturates and benzodiazepines can all be detected in maggots feeding on corpses of drug-related deaths (references within Campobasso *et al.* 2004). The drug levels within maggots are not related to the original level in the corpse, so they cannot be used to estimate pre-mortem drug levels (Tracqui *et al.* 2003).

Roeterdink *et al.* (2004) showed that larvae that had been feeding on or around gunshot wounds had higher concentrations of lead, barium and antimony than control larvae. As larvae developed the lead and antimony were eliminated but the concentration of barium remained fairly constant, thus providing a good indicator of gunshot trauma in corpses, where original evidence of wounds has decomposed or been removed by the larvae themselves.

By molecular analyses of the internal contents of insects, it is possible to obtain a profile of the corpse that can aid investigators in identification of the body (human crab lice Lord *et al.* 1998; dipteran larvae Wells *et al.* 2001a, Linville *et al.* 2004 and Zehner *et al.* 2004; Beetle larvae DiZinno *et al.* 2002). This may be important in cases where no body is present (post-mortem transportation of body from original scene). Insects could link a body back to this scene. If necessary an entomologist

could also prove, by this analysis, that the insect evidence they have based their conclusions on was linked to the corpse in question. Clery (2001) discussed the possibility of obtaining the profile of a rapist from maggots feeding on the genitals of a rape-murder victim. Along with obtaining the semen donor's profile, the researcher also showed that prostate specific antigen (PSA) could be obtained from laboratory maggots feeding on dosed meat. This antigen is confirmatory for semen. Campobasso *et al.* (2003) found however that the success of obtaining a profile decreased with the amount of time the larvae were removed from the human food source.

Insects can also provide information in cases of child abuse and neglect of the elderly as the presence of insects will indicate the length of time children/the elderly have been in unhygienic conditions (Benecke and Lessig 2001, Benecke *et al.* 2004).

1.4 Factors affecting insect evidence

Whilst the methods of PMI estimation and other entomological evidence can be applied in the majority of cases, there are a variety of factors that can affect a forensic entomologist's conclusions. Some factors are presented below. This list (Sections 1.4.1-1.4.8) is not exhaustive.

1.4.1 Effect of Maggot Masses

Temperatures can be raised by the large aggregations of maggots (often known as a larval/maggot mass). Within these masses the temperatures can be raised to above 40°C which is around the lethal temperature for *C. vomitoria* and although the larvae could move from within the maggot mass to the exterior to cool themselves down, this was not observed by Turner and Howard (1992) who noted that high temperatures did not appear to adversely affect larval growth. The increased temperature experienced by the larval masses albeit for short periods would not be accounted for in ADH calculations and thus produce less accurate PMI estimations.

1.4.2 Appearance of Cadavers

Komar and Beattie (1998) illustrated that blowfly maggot masses could disrupt the clothing left upon pigs' corpses in such a manner that it could resemble the state of

undress common in cases of sexual assault on a discovered human body. Maggot masses were capable of moving even tight clothing such as undergarments down the legs of the pigs and in one case completely removed a sock off a pig and moved it 35cm away from the decomposing corpse. This could mislead any investigators discovering the scene after maggot mass activity. The researchers suggest that the presence of many pupal cases around the removed clothing may be an indicator of insect induced clothing artefacts.

Arthropods have also been known to cause post mortem trauma to bodies. In the 19th century a father was accused of having poisoned his child with sulphuric acid following observation of black marks round the child's mouth. These marks were later found to be caused by cockroaches (Klingelhöffer 1898, in review by Benecke 2001). Ants have also been shown to cause post mortem wounds (Campobasso *et al.* 2004). Harada *et al.* (1999) comment on a case of a centipede leaving a subcutaneous cavity in the forearm of a fresh corpse. If the centipede had not been observed leaving the body the trauma may have been thought to be caused by an artificial instrument. Arthropods can not only cause post-mortem injuries but can also remove any diagnostic features of pre-mortem injuries such as gun shot wounds (Roeterdink *et al.* 2004).

Byrd and Butler (1997) showed that there was a 24-hour delay in pupation if movement of *C. rufifacies* larvae is restricted during migration. They note that this will alter developmental timings in cases of corpses wearing tight clothing or that are tightly wrapped, thus providing less accurate estimates of PMI.

1.4.3 Presence of Precocious Larvae

As noted by Smith (1986) it is possible for blowfly females to hold a single egg in the vagina until a suitable oviposition site is found. This egg will be still undergoing embryonic development and so when oviposition finally occurs it often hatches immediately. This is known as a precocious larva. This phenomenon has been noticed in laboratory populations of *C. vicina*, *C.vomitoria*, *L. sericata* and *Calliphora terraenovae* Macquart (reported in Wells and King 2001). Indeed, Wells and King (2001) found 62% of gravid *C. terraenovae* to be carrying an egg within. The presence of these larvae would affect PMI estimates if calculations had been performed solely on the estimated age of these larvae. Using these insects, the PMI

will be overestimated. To avoid this, a large number of insects need to be examined (if possible) to draw conclusions on the PMI.

1.4.4 Effect of Drugs

As discussed in Section 1.3, maggots feeding on corpses containing drugs will ingest these compounds. Experiments have shown that these drugs will have an effect on the developmental lifecycles of the maggots themselves. O'Brien and Turner (2004) indicated that paracetamol accelerated the growth of laboratory *C. vicina* larvae when compared to controls fed on non-drugged liver. Goff *et al.* (references within Musvasva *et al.* 2001) conducted a series of experiments that showed that various drugs altered the rate of development of blowfly positively and negatively. Musvasva *et al.* (2001) observed that the known drug effects on humans couldn't predict the effects of drugs on blowfly. Standard PMI estimations will therefore be inaccurate if based upon insects that have been feeding upon corpses containing drugs.

1.4.5 Differences in Faunal Succession

1.4.5.1 Second Wave of Calliphoridae

Although it is generally accepted that *Calliphora* and *Lucilia* species are early invaders of carrion, they have also been noted to arrive later in the decomposition process. Archer and Elgar (2003) postulated this was due to gravid females arriving in the early successional wave to oviposit/larviposit and non-gravid females and males making up a second wave of Calliphoridae. Non-gravid females need a protein source to complete vitellogenesis and this could come from early/late decay of carrion. Males attend carcasses to mate (they do not require a protein source for testes maturation), therefore they can arrive either immediately or later in corpse decomposition. The appearance of Calliphoridae later in the decomposition process than expected might lead to the underestimation of the minimum PMI. The presence of other arthropods, such as beetles, would provide a more accurate value for PMI in this instance.

1.4.5.2 Burial Conditions

Soil type and burial conditions can also play a part in causing inconsistencies in calculating the actual PMI. Burial leads to different species being present on the corpse. Buried corpses tend to not include the first wave of insects (Calliphoridae),

normally associated with those on the surface, due to the lack of opportunity to lay eggs on the corpse (Bourel *et al.* 2004). However, if a corpse is buried for a length of time and later exposed, the 'normal' waves of insect succession may then occur, thus causing an investigator to grossly underestimate the PMI. Turner and Wiltshire (1999) report a case and a subsequent study carried out in woodland in the south of England where carcasses remained buried for three months before being discovered by scavenging carnivores. This activity exposed the corpses and thus attracted insects. *Calliphora vomitoria*, established as an early invader of carrion, began to lay eggs on a corpse that had been dead for more than 100 days. The conditions of the soil in this area (high proportion of clay) had delayed decomposition so that the corpse was still attractive even after a hundred days post death.

Similarly, wrapping of the body limits access to some of the fauna that would normally arrive and will alter the accepted pattern of faunal succession.

Submerged bodies will also have a different faunal succession. They only include Calliphoridae if any of the body is exposed above the water for an appreciable length of time (Hobischak and Anderson 2002). Haefner *et al.* (2004) indicated that submerged PMI can be estimated using algal growth, but not insect succession as no saprophagous insect food chain appears to exist in the aquatic environment (Anderson and Hobischak 2004).

Introna *et al.* (1998) noted that colonisation of burnt bodies could not occur until the body had cooled sufficiently. A one-week delay was observed on a pig corpse (Catts and Goff 1992). Avila and Goff (1998) burned pigs' carcasses until the body was still recognisable but charred. They did this before exposure to insects (carcasses were kept sterile after death before the burning). They showed that burnt carrion was still as attractive for blowfly oviposition. However, whilst the stages of faunal succession were similar for control and burnt carrion, the timings of some of the waves varied.

Shalaby *et al.* (2000) compared the faunal succession of prone and hanging corpses. The hanging body showed a delay in progression through the stages of decomposition, which they attributed to reduced numbers of dipteran larvae feeding on the carcass

and an inability of ground dwelling invertebrates to access the body. Likewise any feeding larvae falling away from either corpse could not return to the hanging body.

Whilst variations in faunal succession can help an investigator pinpoint the idiosyncratic nature of a particular scene, they could also seriously mislead an investigator when producing an estimation of PMI if the succession is similar but not the same.

1.4.5.3 Regional Variations

It is important to note that the particular species within the faunal succession will vary from region to region. Whilst the succession of families will not vary greatly, e.g. whilst the Calliphoridae will generally arrive first, the sequence of species within the families may vary. *Calliphora vicina* is a dominant primary invader (Hwang and Turner 2005). In the southern USA (Virginia, Tabor *et al.* 2005) *P. regina* is the dominant species in spring and summer but *C. vomitoria* and *Lucilia illustris* Meigen in autumn. In India, Bharti and Singh (2003) noted that *Chrysomya megacephala* (Fabricius) and *C. rufifacies* were the primary invaders after conducting faunal succession experiments. The differences in temperature between areas is reflected in the differences in species. It is therefore critical when using faunal succession patterns to establish minimum PMI, that appropriate species succession data for the particular region is utilised.

1.4.5.4 Potential variation between animal models

Animal models have often been used in forensic entomological research to examine faunal succession under varying conditions. Most experimenters use the domestic pig (*Sus scrofa* Linnaeus) as they have equivalent thoracic size to humans, their skin is also relatively hairless and they have similar gut flora, as they are also omnivores (Komar and Beattie 1998). Putrefaction, the post-mortem destruction of soft tissue during the active decay stage, is caused by the action of endogenous bacteria and enzymes. The majority of these bacteria are from the alimentary canal and therefore to eliminate potential variability in decomposition rates an experimental model with a similar diet should be chosen. Whilst is assumed (after Niezabitowski 1902 in Benecke 2001) that human corpses have the same pattern as all other animal corpses

there is the potential for variation in succession if smaller animals with fur for instance are used.

1.4.6 ADD/ADH Model

The model, as described in Section 1.1.1, only truly applies to the temperatures where the relationship between temperature and developmental rate is proportional. At the temperature extremes the developmental rate is no longer proportional. If insects feeding on a corpse have experienced these extreme temperatures they will not accumulate degree hours as described by the ADH model and estimates of PMI will consequently be inaccurate (Ames and Turner 2003). The temperature ranges for which the ADD/ADH model applies will vary between species. An investigator would need to take into account any extremes of temperature experienced – these could be natural or artificial (refrigeration).

Another problem with use of the ADH model is the derivation of the minimum developmental threshold. This threshold is difficult to determine empirically as insects can survive at low temperatures where development slows to near zero. The minimum developmental threshold is most commonly achieved by extrapolating a graph of low temperatures where development is still clear, to the x-axis where development is zero. There is a lack of definitive temperature thresholds for species and for a few species discrepancies exist within the literature (e.g. for *C. vicina* thresholds of 6°C (Kamal 1958, data within Higley and Haskell 2001); 2°C (Davies and Ratcliffe 1994; Hwang 2004) and 1°C (Donovan, Hall and Turner *unpublished work*, personal communication)). Due to the nature of the ADD/ADH calculation the use of varying minimum threshold temperatures will affect the minimum PMI estimation.

1.4.7 Effect of different body tissues

Recent work has indicated that the types of body tissue on which the larvae have been feeding in the laboratory may influence their developmental rate (Kaneshrajah and Turner 2004, Clark *et al.* 2005 *in press*). Kaneshrajah and Turner indicated that *C.vicina* developed slower on liver. Clark *et al.* also found that a diet of liver produced smaller *L. sericata* larvae and eventually adults and developed at a slower rate than those on heart and lung. Green (1951) tried to assess the tissue preferences of blowfly by examining the meat choice of blowfly in slaughterhouses. He noted the

tissue from the gut appeared most attractive to blowfly. The problem with using slaughterhouse meat samples is that they will not represent the natural 'choices' of blowfly on a corpse, as the meat in slaughterhouses is more easily accessible than it would be on a corpse with no external wounds. More recently Klotzbach *et al.* (2005) noted where larvae were located on discovered human corpses in Germany. They found that the immediate area of infestation on a fresh corpse to *C. vicina* and *L. sericata* was the face and genital region (depending on accessibility due to clothing). Once the body had started to decompose larvae were found all over the body.

Developmental rates for different species established within the laboratory will be based upon one specific tissue type, thus may actually produce results not applicable to larvae that have fed on other tissues or indeed several tissue types. This may lead to inaccuracies in PMI estimations.

1.4.8 Problems with Identification

Forensic investigators depend upon accurate identification of arthropod evidence. Developmental growth rates and habitat occupancy differ between species and therefore inaccurate identification will affect an entomologist's conclusions.

Traditionally identification is based upon morphological differences between species. Often this is carried out by rearing the larvae/pupae from a crime scene up to the adult stage. The adult stages of most species are less difficult to distinguish than the immature forms. Occasionally rearing will not be possible if for instance the evidence has not been collected properly often leading to failure to develop or if the estimation for PMI is required quickly. Errors in identification will lead to incorrect development data being used and thus produce inaccurate PMI estimations.

It is also possible that there are differences between the biology of populations within the same species. Organisms become adapted to their niches, especially if a species has a particularly wide range covering various habitat types. Entomologists usually use lifecycle data that has been obtained from laboratory populations that may not be applicable to all populations. Again this may lead to inaccuracies with minimum PMI estimations. Populations cannot be differentiated from each other as there are often no morphological differences between them.

1.4.9 Accurately determining insect age

One method of calculating the minimum PMI using the ADH model is to place the discovered insects in a controlled temperature environment and note the time of adult emergence. However this is not always practical as completion of development is time consuming. Also, insect evidence is not always collected from the scene by those aware of the entomologists' requirements and can therefore be ill handled – thus not allowing further development. .

Another method is to determine the developmental stage morphologically and use the appropriate ADH value required to reach this stage. As demonstrated in Figure 1.1 the pupal stage of Calliphoridae can be relatively long in duration, thus lowering the accuracy of the PMI estimation by simply using the ADH to pupal stage value.

Isomegalen-diagrams are limited to the larval stages as pupae do not change in length. They are also derived using constant laboratory temperatures, which are often not good comparators to fluctuating environmental temperatures. Conditions, such as increased competition for the food source, have been shown to stunt larval growth (Smith and Wall 1997b, Hwang 2004). This length change will lead to error in determining insect age and consequently lead to inaccuracies in PMI estimation.

Aim of the overall study

The aim of this work was to focus on two of the challenges facing forensic entomologists as described in Section 1.4, namely accurate identification of insect evidence and more precise age determination of the immature stages of forensically interesting dipteran species.

Molecular techniques were employed to attempt to resolve these two problems. This work aimed to locate molecular markers to not only distinguish between species but also between geographical populations of blowfly that resemble each other morphologically. As discussed previously, precise identification of insects is very important for producing accurate conclusions from the evidence. Whilst morphological differences exist between species, these differences are not present between populations. Populations therefore require an alternate method of identification.

This study then focussed on defining molecular markers that are present at different timepoints during the development of insects to consequently be utilised in determining the age of immature forms.

It is intended that the identification of genetic markers will form the basis on which a kit can be developed for use in forensic laboratories to allow rapid identification and aging of forensically interesting dipteran species.

Calliphora vicina and *C. vomitoria* were chosen as experimental models for this study as they are two of the most prevalent species of forensic interest in the UK and are often the first invaders of carrion. Corpses in the UK are usually discovered relatively quickly especially in urban situations and therefore Calliphoridae will often be the only insect evidence available.

Chapter 2

Methodology for Specimen Collection

The *Calliphora* specimens used in this work were collected and reared as described in the following sections.

2.1 Areas for Specimen Collection

Several areas were used as trap sites (Figure 2.1). Table 2.1 contains a description of each of the trap sites (personal observations, personal communications, Hwang (2004)) along with OS grid references (www.getamap.ordnancesurvey.co.uk). Calculation of the distance between all of the trap sites in this study was done using the OS grid references (Table 2.2).

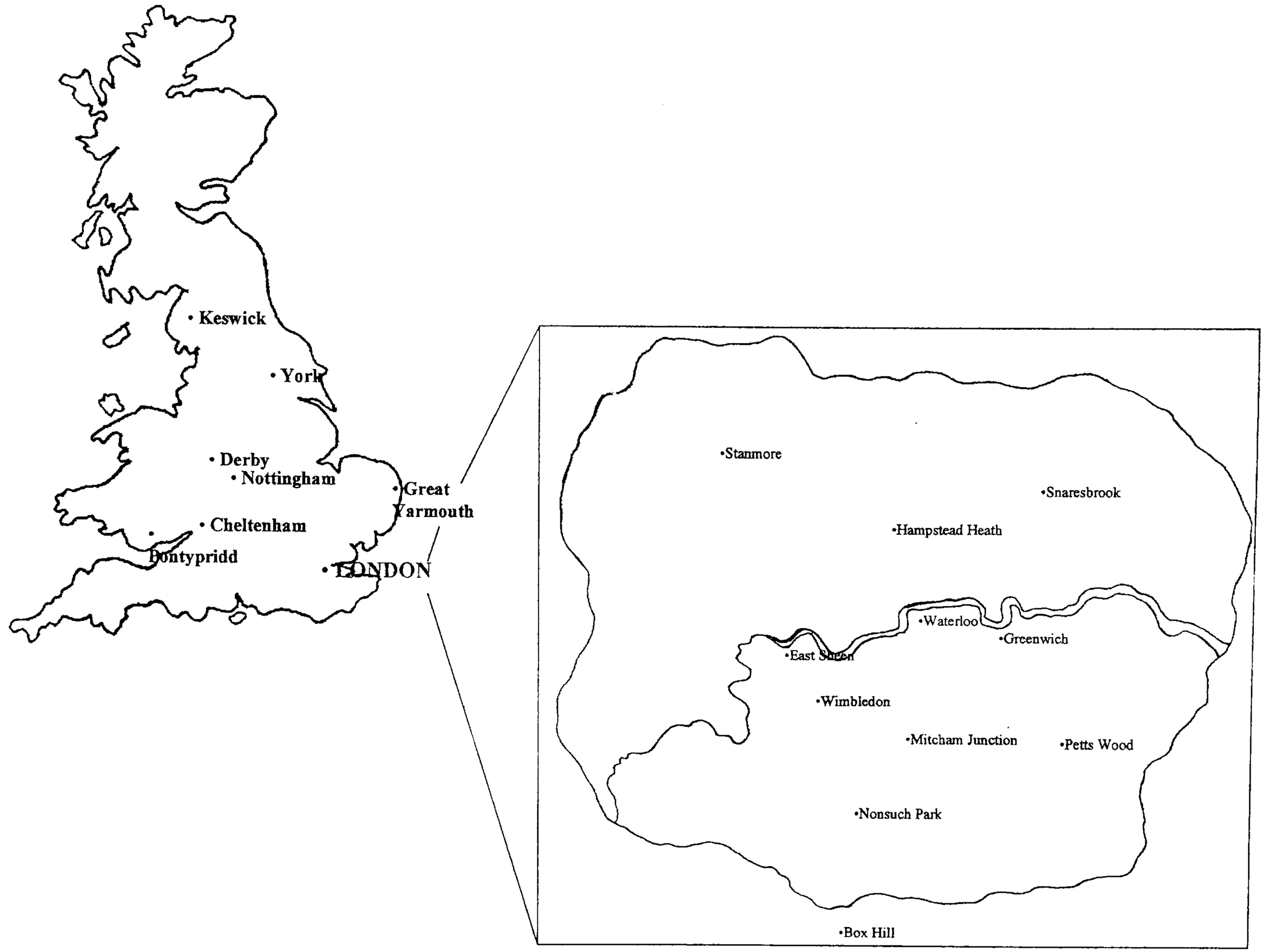


Figure 2.1. Sites of traps within England and Wales. Insert shows trap sites within London with relation to the River Thames and the M25 boundary.

Table 2.1. Description of trap sites within England and Wales along with OS Grid references.

Sample Site	OS Grid Reference[b]	Description	Observation of site and flies trapped by
Wimbledon Common	TQ 218 719	Dense deciduous woodland	Chung-chi Hwang[a]
Wimbledon Centre	TQ 249 704	2m from ground level, balcony in urban area beside houses with gardens	Carole Ames
Ripley (Derbyshire)	SK 409 512	Rural site, non shaded grassland, few trees.	Hélène Le Blanc
Great Yarmouth	TG 525 080	In cemetery, within town centre	Carole Ames
Pontypridd	ST 082 882	Overgrown garden in small town. Adjacent to woodland	Carole Ames / Matthew Ames
Cheltenham Urban	SO 948 197	Large Garden alongside others, on outskirts of town	Carole Ames
	SO 938 243	Small garden alongside railway and stream	Steve Tann-Ailward
Leckhampton Hill (Cheltenham)	SO 956 189	Semi natural grassland alongside woodland.	Carole Ames
York	SE 602 514	Small patch of grass alongside river within city	Carole Ames
Keswick	NY 261 229	Two traps – one in town carpark shaded	Carole Ames

		by bushes. The other in long grasses alongside woodland and large lake	
Nottingham	SK 561 377	Patch of grass alongside carpark. Fairly shaded	Carole Ames
Mitcham Junction	TQ 282 679	Small woodland by Common near housing.	Chung-chi Hwang[a]
Nonsuch Park	TQ 231 644	Woodland surrounding park	Chung-chi Hwang[a]
Snaresbrook	TQ 403 887	Parkland	Chung-chi Hwang[a]
Waterloo	TQ 311 803	Very urbanised area. Few gardens in area.	Chung-chi Hwang[a]
Box Hill	TQ 178 517	Shaded woody valley	Chung-chi Hwang[a]
Stanmore	TQ 174 929	Dense woodland near housing	Chung-chi Hwang[a]
Petts Wood	TQ 440 668	Woodland alongside housing	Chung-chi Hwang[a]
Greenwich	TQ 387 768	Small woodland next to heath and parkland	Chung-chi Hwang[a]
East Sheen Common	TQ 194 749	Woodland	Chung-chi Hwang[a]
Hampstead Heath	TQ 274 862	Parkland alongside small woodland	Chung-chi Hwang[a]

[a] Description précised from Hwang (2004)

[b] Grid References taken from www.ordnancesurvey.co.uk

Table 2.2. Distances (km) between *Calliphora* trap sites within England and Wales. Distances calculated using OS Grid Reference system.

Distances between Sample Sites (km)	Wimbledon	Wimbledon Common	Derby	Great Yarmouth	Pontypridd	Cheltenham	Leckhampton	York	Keswick	Nottingham	Mitcham Junction	Nonsuch Park	Snaresbrook	Waterloo	Box Hill	Stanmore	Petts Wood	Greenwich	East Sheen Common
Wimbledon																			
Wimbledon Common	3																		
Derby	197	199																	
Great Yarmouth	189	188	216																
Pontypridd	214	217	210	365															
Cheltenham	136	139	139	272	92														
Leckhampton	133	136	139	270	95	3													
York	286	288	102	240	304	241	241												
Keswick	402	405	207	391	335	311	312	152											
Nottingham	178	181	20	199	210	133	132	114	226										
Mitcham Junction	7.5	4	203	187	221	143	140	292	408	184									
Nonsuch Park	7.6	6	204	193	216	140	137	294	409	186	6								
Snaresbrook	25	24	190	164	232	149	146	275	397	171	24	30							
Waterloo	13	12	193	176	223	142	139	280	399	174	13	18	12						
Box Hill	21	20	214	206	213	141	138	305	418	196	19	14	43	32					
Stanmore	21	24	176	177	209	125	123	265	381	157	27	29	23	19	41				
Petts Wood	23	19	211	178	237	158	155	297	417	192	16	21	22	19	30	37			
Greenwich	18	15	200	174	231	150	147	286	406	181	14	20	12	8	33	27	11		
East Sheen	4	7	193	188	212	132	130	283	398	175	11	11	25	13	23	18	26	20	
Hampstead Heath	15	16	186	175	219	137	134	274	392	167	18	22	13	7	36	12	26	15	14

2.2 Specimen Collection Methods

Specimens were caught by using either a 'sticky trap' or two variations of the Irwin trap (Erzinçlioğlu 1996). It has been previously shown that baited traps bias the species and relative numbers of blowfly caught (MacLeod and Donnelly 1962). However, this is irrelevant for this study as only the blowfly population genetic structure is to be examined from these insects; no conclusions of population size or proportions of species will be drawn from the numbers caught. It was therefore decided that the following baited traps would be utilised.

2.2.1 'Sticky Trap'

This trap consisted of a piece of adhesive paper (22 x 30cm FLYSON, Nordic Star Ltd, Frome, UK) upon which was placed a 23 x 30cm piece of wire mesh. This mesh provided support for the adhesive paper and allowed ease whilst handling (Robacker and Heath 2001). This was then covered by an inverted plastic basket (20 x 28 x 11cm). The basket was modified slightly to allow greater access to flies by removal of parts of the sides. The trap was secured in position by the use of two 20cm nails (Figure 2.2).

Figure 2.2. Sticky Trap. Trap consisted of an upturned plastic basket fixed upon adhesive paper that was supported by wire mesh. Cuts in the sides of basket allowed entry of flies. Trap was baited with pig liver and sodium sulphide solution.

The trap was baited using 15-20g of commercially obtained pig liver plus a few drops 20% (w/v) sodium sulphide solution (Sigma) in a 8 x 5.5 x 1.5cm weighing boat. This

was shown to successfully attract *Calliphora* species (Hwang 2004). Sodium sulphide solution releases hydrogen sulphide gas on contact with water in the air. Fisher *et al.* (1998) also indicated that the addition of sodium sulphide to fresh bait increased the mean catch per trap. Several drops of sodium sulphide solution were further placed over the adhesive paper.

After a few hours (the actual time was dependent on weather and trap location conditions), when a suitable number of flies had become fixed to the paper, the trap was disassembled and the paper and wire mesh placed in a sealed bag for transportation to the laboratory where the bag was placed in the freezer until required. Of the three traps used in this study, this was the quickest to assemble and the easiest to transport.

2.2.2 Funnel Trap

This trap is based upon the Irwin trap, which uses the phototrophic instinct of most insects to move towards the light along with the principle most flies being incapable of flying back down towards the ground (Erzinçlioğlu 1996). It was constructed by the author based upon diagrams in Borror *et al.* (1989) (Figure 2.3).

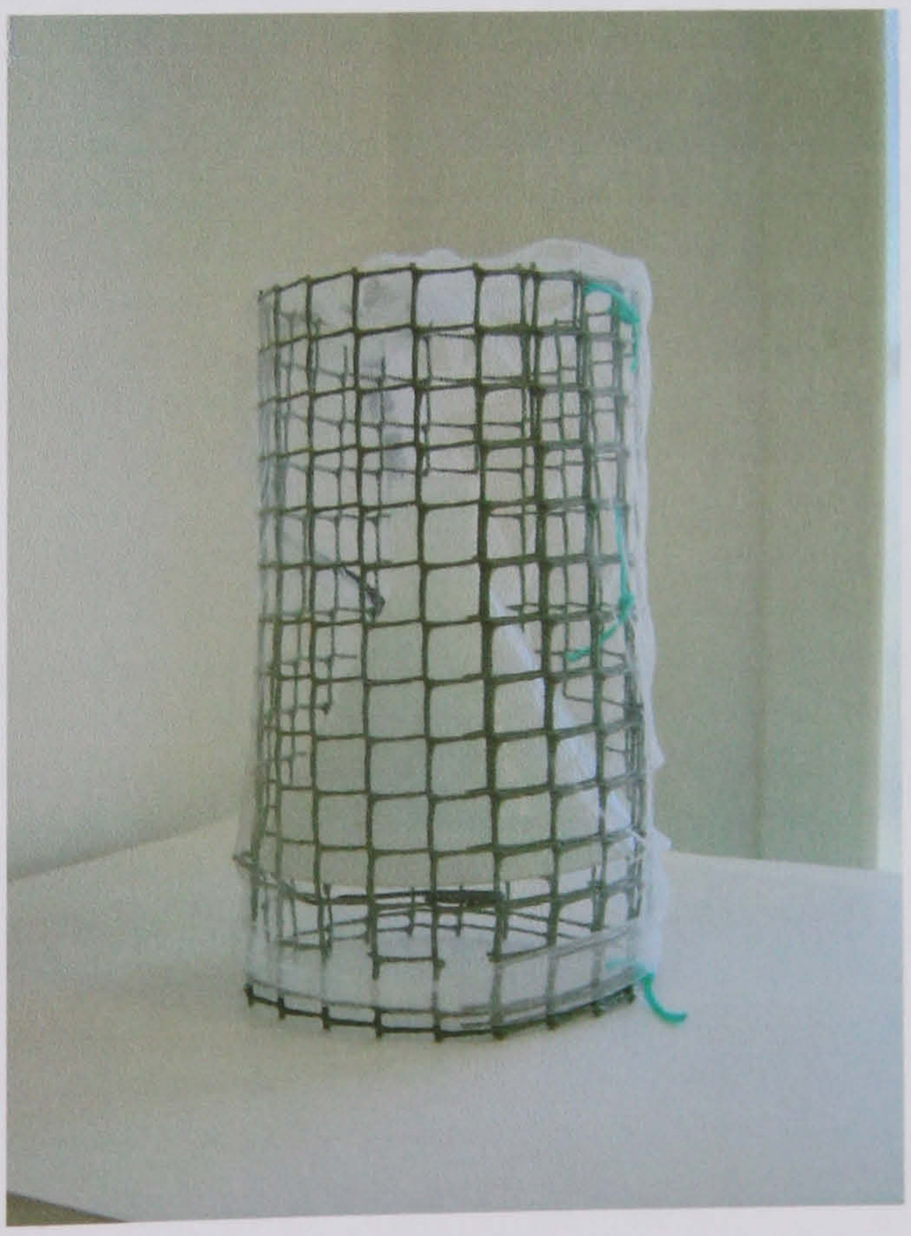


Figure 2.3. Funnel trap. This trap consists of an upturned funnel within a cylinder of plastic mesh covered with gauze. The trap was baited with pig liver at the base directly under the funnel.

This trap consisted of an inverted funnel within a cylinder of plastic flexible mesh (46 x 26 cm). Apart from the base (to allow flies to enter) the trap was covered in gauze to trap any flies that, after being attracted to the bait, flew upwards through the inverted funnel.

This trap was baited with pig liver and 20% (w/v) sodium sulphide solution was added to the meat. This trap has the advantage over the sticky trap as the flies remained alive and so could be used to propagate laboratory populations.

2.2.3 'Bottle' Trap

This trap was designed and constructed by Dr C. Hwang (personal communication, Hwang (2004)) and is also similar to the Irwin trap.

The top third of a 1.5l plastic drinks bottle is placed within a similar bottle. Square holes (1 x 1cm) were made in the sides of the bottle at the base to allow access to the bait for flies. This trap was again baited with pig liver (15-20g) placed within a weighing boat (8 x 5.5 x 1.5cm). A small glass bottle (15ml) containing 20% (w/v) sodium sulphide solution (Sigma, UK) and topped with gauze was also placed in the base of the trap. Flies arriving at the bait fly upwards and get trapped in the top section of the trap. Holes (2mm diameter) had been punched into this section to let air into the trap (Figure 2.4). Similar to the funnel trap, this also allows flies to remain alive and intact. This trap has the advantage over the funnel trap because it was very simple to assemble and position. The funnel trap is best in locations where the trap can remain for longer periods of time.

Any flies caught in this trap and the funnel trap were removed and placed in either 95% ethanol (BDH) within a tube or in a vented container for transportation back to the laboratory. They were then stored at –20°C until use or kept alive for addition to laboratory populations.

Figure 2.4. Bottle Trap. This trap consists of a shortened plastic drinks bottle inserted within another. Holes at the base allow flies access. The trap was baited with pig liver and sodium sulphide solution.

2.3 Laboratory Fly Populations

Laboratory *Calliphora* populations were established either from adult flies caught or reared from eggs deposited on the liver bait in the traps.

2.3.1 Laboratory population conditions

Populations of *C. vicina* and *C. vomitoria* from different locations were raised and maintained in gauze-covered cages (23.5 x 38 x 27 cm) at room temperature (Figure 2.5). Flies were supplied with granulated sugar and water *ad libitum*. Each cage contained approximately 100 flies to avoid overcrowding (Saunders 1997).

Flies were harvested and killed by low temperature (-20°C). Knipling (1957) indicated that 100% mortality occurred in the housefly after 30 min at -20°C. Previous observations indicated that exposure to -20°C killed both *C. vicina* and *C.vomitoria* adult flies after approximately 15 min. Specimens were either used immediately or placed in universal tubes containing 95-99% ethanol and kept at –20°C until needed.

Figure 2.5. Laboratory fly cage. Cages consisted of 23.5 x 38cm trays. Attached to these was 52 x 35.5cm piece of plastic mesh to form a 'dome' shaped top. The whole trap was then covered in gauze with tied knots at the sides for access to the cage.

Non-gravid females require a protein source to complete vitellogenesis, as mentioned previously, so pig liver was -placed in the cages after the introduction of new adults. When immature forms were required, fresh butchered pig liver was cut into 2 x 3 x 2cm pieces and placed in a weighing boat at the bottom of the cage. Females massed upon the liver and oviposition occurred within one hour of feeding. Once eggs were present, usually in batches of approximately 250, the weighing boat was removed from the cage. It was placed in a plastic container (7 x 8 x 11cm) with a plastic lid (Hot Form Productions, UK). Multipurpose compost was placed in a 2cm layer at the bottom of each to act as a medium for post feeding larvae to bury into for pupation (Figure 2.6).

Figure 2.6. Container for rearing larvae. Holes pierced in the lid allowed air to circulate. Compost at base of container was for wandering larvae to bury into to pupate.

According to Smith (1986) larvae should be reared in moist conditions, but closed containers will cause asphyxiation from a build up of carbon dioxide and encourage the growth of mould. To avoid this ten or more puncture holes (approximately 1-2mm diameter) were made in the lid of each plastic container employed. Holes were kept small as it was noted from previous work that *C. vomitoria* larvae will leave the food source periodically during the first and second larval stage and can escape the containers if large holes are made (personal observation).

Containers were kept at 20°C in a cooled incubator (LMS, UK). Larvae were killed in boiling distilled water and either used immediately or placed in universal tubes containing 95-99% ethanol (BDH, UK) and kept at –20°C.

2.4 Species identification

To confirm the species of both adult and immature forms before experimentation began they were characterised morphologically, using a key provided by Smith (1986).

For *C. vicina* and *C. vomitoria* adults, the basicosta of the wing of *C. vomitoria* is always black compared to *C. vicina* on which it can vary in colour from yellow, orange through to dark brown (Figure 2.7).

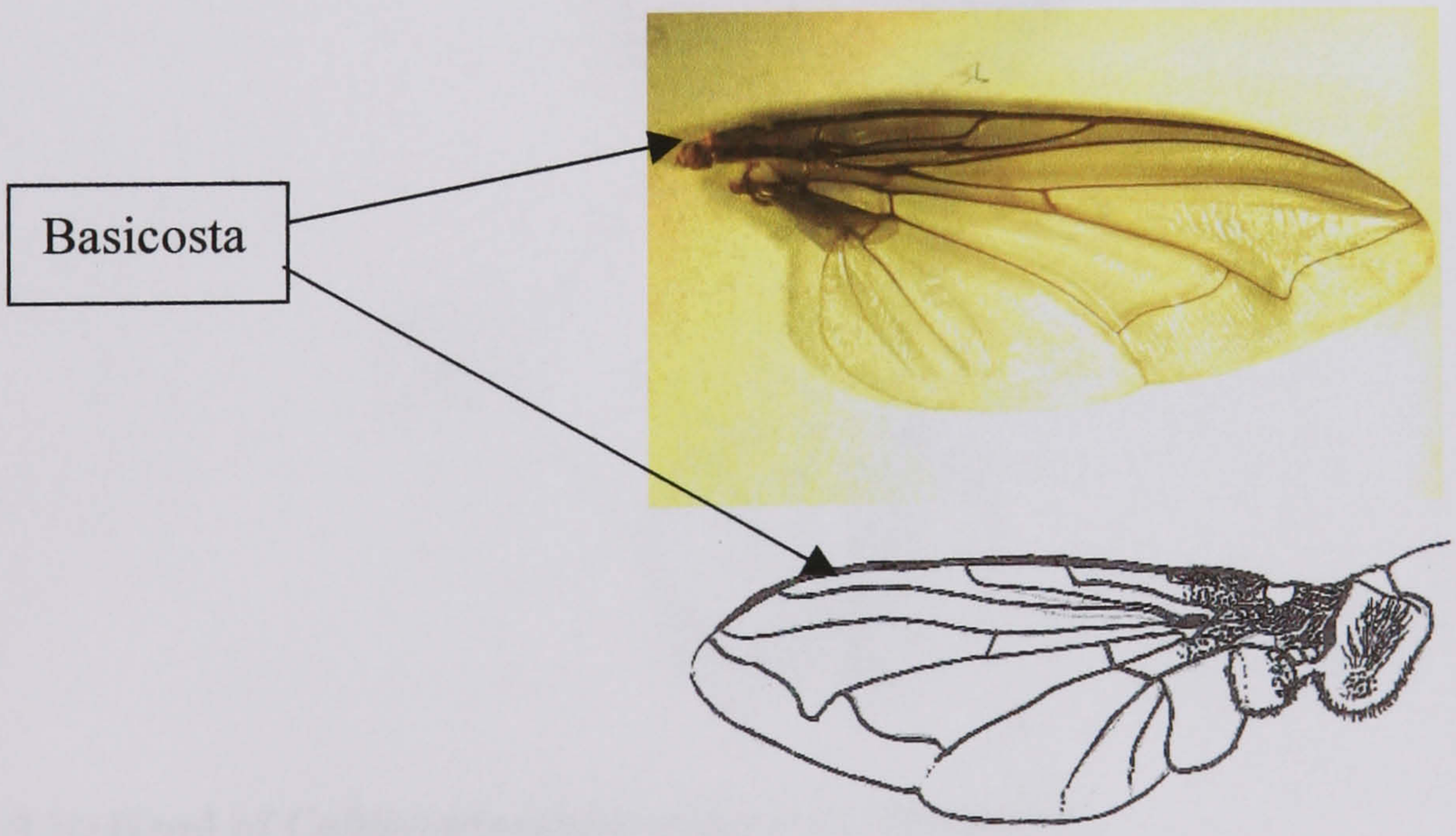


Figure 2.7. Wing of *C. vicina* to demonstrate position of the basicosta.

Calliphora vicina adults have an orange anterior spiracle whereas the anterior spiracle on *C. vomitoria* adults is dark (Figure 2.8).

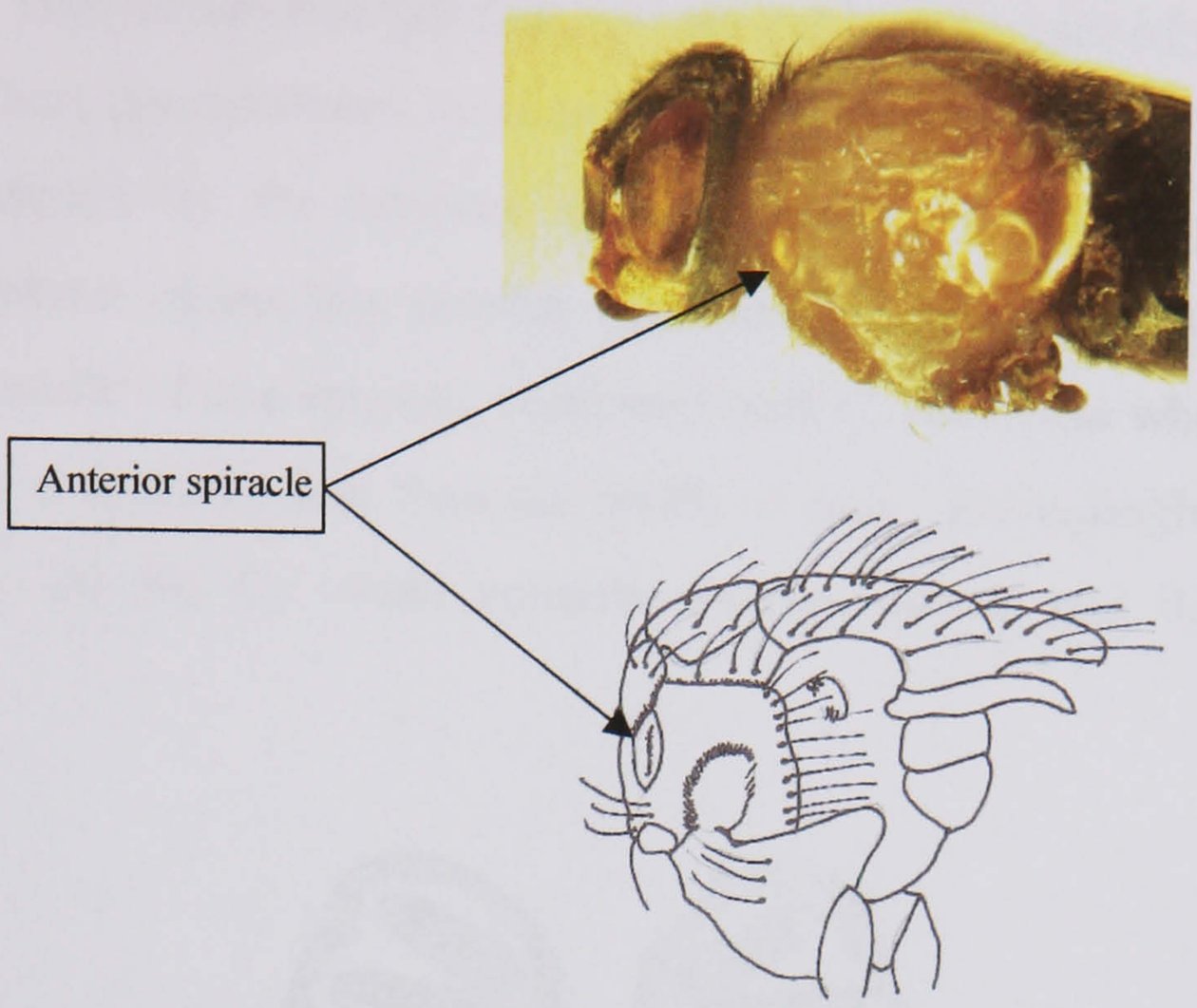


Figure 2.8. A) Photograph of head, thorax and upper abdomen of *Calliphora vicina* (wings and legs removed) and line drawing to illustrate position of anterior spiracle.

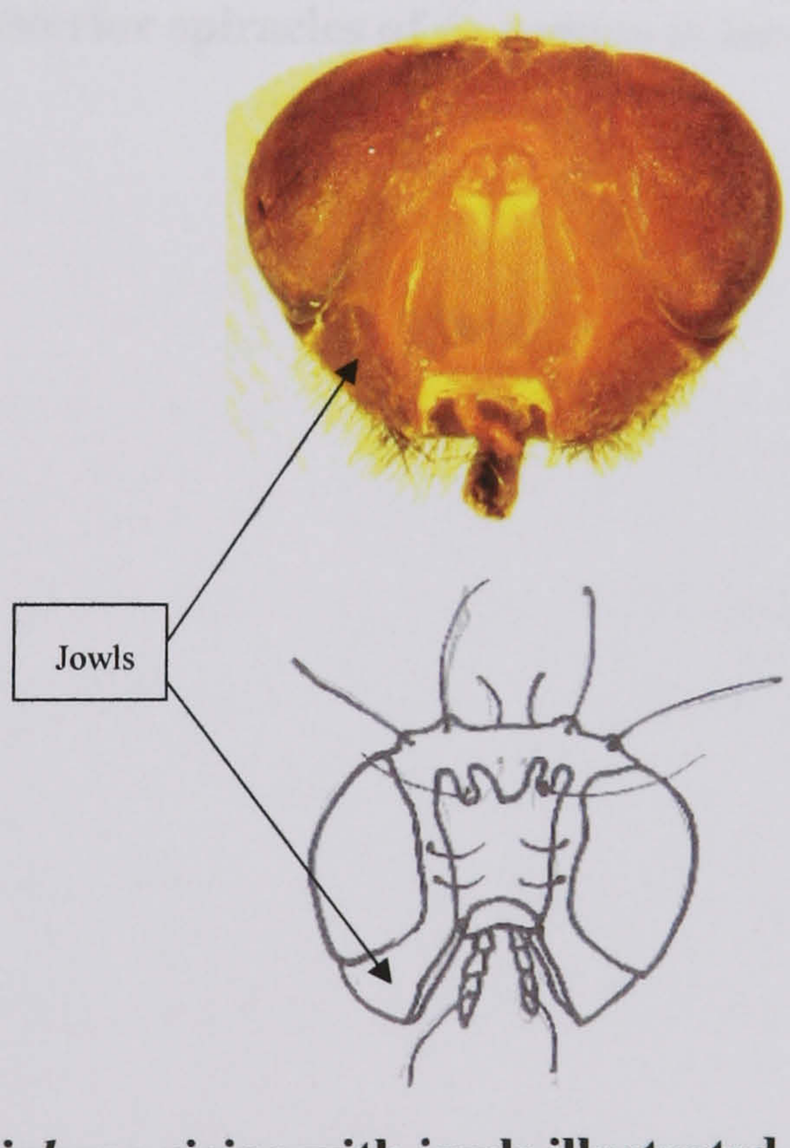


Figure 2.8 B) Head of *Calliphora vicina* with jowls illustrated.

The hairs on the jowls are black on *C. vicina* and orange on *C. vomitoria*. The jowls (the anterior two thirds) are orange coloured on *C. vicina* (Figure 2.8) whereas *C.vomitoria* has dark jowls.

For immature forms, morphological identification is less clear. Hall and Robbins (2005) have shown that there appears to variation at a local level in morphometric measurements. They concluded that first and second larval stages of *C. vicina* and *C. vomitoria* were best discriminated by examination of the mouthparts. The third stage can be differentiated by the between spiracle distance divided by width of one spiracle. *Calliphora vicina* has smaller spiracles that are separated by a distance greater than the width of one spiracle, compared with *C. vomitoria* where the spiracles are separated by a distance less than the width of one. Erzinçlioğlu (1985) quotes values of 1.10-1.20 for *C. vicina* spiracle distance factor and 0.69-0.80 for *C. vomitoria*.

Figure 2.9. Diagram of posterior spiracles of *C. vomitoria* larvae. Drawn from diagrams in Smith (1989).

Part I

Location of molecular markers to aid in the identification of forensically interesting dipteran species

Chapter 3 Introduction to the identification of Calliphoridae

As discussed briefly in Section 1.4.8, the use of entomological evidence depends upon accurate identification of insect species. As developmental growth rates and geographical distribution can vary considerably between species and even within species, inaccurate or uncertain identification can harm or hinder an investigation – consequently leading to miscarriages of justice (Benecke and Wells 2001).

Insects recovered as part of the evidence from crime scenes is usually placed in controlled laboratory conditions and allowed to progress to adult forms for identification. Traditionally this identification was based upon morphological differences between species.

However, occasionally entomologists do not get access to corpses *in situ* but are given preserved entomological evidence at a later date, often collected from the scene of a crime by inexperienced personnel and so the rearing of insects is not possible. Harvey *et al.* (2003) also stress that rearing insect evidence removed from a crime scene in fact compromises the integrity of the evidence as it changes it from the form found. Another problem is that the rearing of immatures takes time. Police and the judicial system often require swift results especially if the evidence provides intelligence for further investigation.

Accordingly, species identification should ideally be carried out upon whatever life stage is presented and in whatever state of preservation.

In some instances, morphological species identification can be difficult, even for specialists. One reason is that the order of Diptera consists of many species and therefore there are very few experts for a whole insect order. Different regions have different fauna and there are not always identification keys that cover all the species within a particular area. Wallman (2001) produced a key for adult blowflies in Southern Australia but the author notes that this key is not comprehensive enough

even to be used in the rest of Australia.

Another reason is that the immature stages of many forensically important dipteran species are very difficult to distinguish as they vary by small morphological differences. Indeed, Greenberg and Singh (1995) after Erzinçlioğlu (1989) indicated that variability between published descriptions of Calliphoridae eggs existed. They showed that this was not due to different methods of sample treatment before observation, although this is another factor for consideration. They found that *C.vomitoria* eggs were indistinguishable from *C. vicina* even though Erzinçlioğlu (1989) had shown clear differences in the egg median area. Similarly, Erzinçlioğlu found that the median area was interrupted along its length only in eggs of *Lucilia* species whereas Greenberg and Singh (1995) noted this phenomenon in some *C.vomitoria* egg samples. These researchers found diagnostic differences between eggs of the same species from different parts of the world for *L. sericata*. They also showed intrapopulation differences in *Protophormia terraenovae* (Macquart), *C.vicina* and *P. regina*.

Larvae of the genus *Chrysomya* can be easily distinguished from other blowfly larvae by the fact they are 'hairy' (they have a row of tubercles on each segment) but it is relatively hard to separate the species within the genus morphologically (Wells and Sperling 1999).

Ideally other methods need to be used to identify forensic insect evidence, either to refute or confirm identification by morphological differences.

3.1 Recent approaches to identification

The challenge of species identification has been highlighted in the literature and recent research has involved other methods for distinguishing between carrion fauna. This research has highlighted differences between either amino acid or deoxyribonucleic acid (DNA) sequences. DNA is a polymer of nucleotides, which consist of a phosphate group, deoxyribose sugar and one of four bases – adenine, guanine (A, G; purines), cytosine and thymine (C, T; pyrimidines). The genetic code is a triplet code, the order of bases within each triplet (codon) codes for a specific amino acid. Thus, it is the order of bases within genes that determines the sequence of amino acids during protein synthesis and thus the eventual protein produced. As there are 64 possible

codons and only 20 amino acids, several codons code for one amino acid hence the code is degenerate.

An advantage of using molecular markers for identification is that they are relatively insensitive to the state of preservation of the sample. It is not necessary to maintain the samples in a living state. This insensitivity to preservation may be especially valuable in cases where it is necessary to use old insect evidence. Previous research has utilised the following methods.

3.1.1 Immunological

These methods are based on the antigenic nature of proteins. Antibodies for a protein specific to a species are generated in a host (often rabbit or mouse) and are then linked to an enzyme that catalyses a visible reaction, usually a colour change in the presence of the specific antigen.

Figarola *et al.* (2001) have developed an antibody-based enzyme linked immunosorbent assay (ELISA) which allows the rapid identification of eggs, larvae, pupae and adults of *Cochliomyia hominivorax* Coquerel to distinguish them from *C. macellaria* and other species. The use of ELISA may not be sufficiently discriminating to distinguish closely related species. Also, initial use is expensive and time consuming as appropriate antibodies have to be raised to use in assays.

3.1.2 Biochemical

Some researchers have used biochemical differences between and within species. Byrne *et al.* (1995) studied the composition of cuticular hydrocarbons (which are ultimately determined by genotype) and used the hydrocarbon profiles to separate *P. regina* by gender and location.

3.1.3 Allozymes

Allozymes are different enzyme alleles at a specific locus and therefore reflect mutations in coding regions of DNA. These mutations have modified the net charge of an enzyme, so that the different enzymes can be separated electrophoretically. Wallman and Adams (2001) used allozyme markers to differentiate four forensically important Southern Australian blowflies. These markers could be used to identify unknown larvae by comparison with adult allozyme profiles. Taylor and Peterson (1994, 1995) used a series of allozymes to examine the population genetics of *C.*

macellaria and *C.hominivorax*.

However, only one sixth of amino acid substitutions that produce new allozymes can be differentiated electrophoretically. A lot of potential variation is overlooked by the use of these allozyme markers. Also, as allozymes are the functional products of inherited nuclear genes, they will undergo recombination and can also be subject to selection, which can result in reduced variability.

Allozyme markers are often expressed only in particular life stages. While this could be valuable in distinguishing between life stages it does not benefit species identification, where markers should apply to any stage found on a corpse. They are a relatively cheap method of identification but they are easily degraded in material that is not live or deep frozen (Sperling *et al.* 1994).

The above techniques rely upon differences in protein, which naturally are reflections of differences in coding DNA. It therefore follows that a better differentiation method would focus on variation in DNA sequences. This would allow greater levels of polymorphism to be detected.

Forensic DNA techniques established for human identification have been adopted by entomologists for use on insect DNA.

3.1.4 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism procedure (RFLP) involves the cutting of DNA with restriction enzymes.. Restriction enzymes cleave double stranded DNA at all sites that match a specific sequence. These fragments are then separated electrophoretically. .

Restriction enzymes can be used to cut either side of a minisatellite locus (a repeat sequence of greater than 15 base pairs in length). The repeat fragments are separated to produce a 'fingerprint' (Jeffreys *et al.* 1985).

The disadvantage with using the RFLP method is that it requires a large amount of template DNA and can only effectively be used on large insects (Schlipalius *et al.* 2001). It is not always the case that whole samples are removed from crime scenes and not enough DNA may be present to conduct RFLP successfully.

The introduction of the automated polymerase chain reaction (PCR) (Saiki *et al.* 1985, 1988) for the amplification of small amounts of template DNA has greatly extended the tools for identification open to entomologists.

PCR depends on oligonucleotide primers hybridising specifically to flanking regions of an area of interest within a DNA template. These primers are used to initiate DNA synthesis *in vitro* by a thermostable DNA polymerase from the bacterium *Thermus aquaticus* Brock and Freeze (*Taq* DNA polymerase). This polymerase remains stable at the temperatures required to denature the DNA (94°C to 95°C). Samples are placed in a thermal cycler that performs a series of temperature cycles during which the DNA template is denatured, so that the double strands separate, primers are annealed and extended by addition of deoxynucleotide triphosphates (dNTPs) catalysed by the *Taq* DNA polymerase. Repeated cycling amplifies the region between the primers.

Development of the PCR method has had a major effect on DNA systematics as it is faster and easier to amplify DNA than cloning. A major advantage of PCR is that the method can be used to obtain sequences from degraded nucleic acid sources such as alcohol-preserved tissues (Junqueira *et al.* 2002) or occasionally from fossil specimens (Capelli *et al.* 2003). The disadvantages of PCR are that the flanking sequences must be known for primers to be designed. Also, *Taq* DNA polymerase is expensive and only relatively short sequences can be amplified directly.

3.1.5 PCR-RFLP

Restriction enzymes can be used on PCR products. Sperling *et al.* have produced restriction site maps for *C. vicina*, *C. vomitoria* and *Eucalliphora latifrons* Hough (Sperling *et al.* 1994). Following Sperling *et al.*, Schroeder *et al.* (2003) produced maps for *C. vicina*, *C. vomitoria* and *L. sericata*. Hale and Singh found greater differentiation between worldwide populations of *Drosophila melanogaster* Meigen using restriction digests than was evident with allozyme assays (Hale and Singh 1987).

3.1.6 Random Amplified Polymorphic DNA

One technique that has been adopted by entomologists is random amplified polymorphic DNA (RAPD) analysis (Williams *et al.* 1990). This consists of the amplification of DNA sequences of a large number of sites throughout the genome,

using arbitrary oligonucleotide primers under low annealing temperatures. The potential result is a DNA fingerprint specific to species. Stevens and Wall (1995, 1996 and 1997) successfully used RAPD to differentiate between and within *L.sericata* and *L. cuprina* samples. This technique, whilst being very quick and simple (there is no requirement to design primers for specific genes), has been shown to have poor reproducibility from laboratory to laboratory and between specimens (reviewed by Black 1993). Whilst Benecke (1998) experimented with different PCR machines and template DNA concentrations using standardised PCR reaction beads, he did not consider the effect of different methods of DNA extraction (leading to variation in template quality) or the conditions the samples were raised in (RAPD can distinguish those samples raised in sterile conditions but cannot account for the contamination from extraneous DNA sources e.g. bacteria). To utilise RAPD techniques effectively, protocols from sample storage through to analysis of banding patterns would need to be standardised.

3.1.7 Sequencing

Perhaps the best method for identifying inter-and intra-specific variation is to actually determine the sequence of base pairs of certain amplified DNA regions. This therefore allows both synonymous (causing no amino acid change) and non-synonymous (causing amino acid change) substitutions to be identified. It is this method that many recent studies have adopted.

Nucleic acid cycle sequencing is based upon the dideoxynucleotide chain-termination method of Sanger *et al.* (1977). It utilises a polymerase reaction to amplify labelled DNA that is complementary to the target DNA. An appropriate primer molecule is annealed to a complementary single-stranded segment of DNA in the presence of dNTPs and dye-labelled dideoxynucleotide triphosphates (ddNTPs). DNA synthesis is initiated at the annealed primer and continues until chain growth is terminated by incorporation of one of the four dye labelled ddNTPs. ddNTPs do not contain the 3' hydroxyl group required for further addition of dNTPs, thus stopping DNA synthesis. A genetic analyser is employed to separate the fluorescently labelled DNA fragments, often by capillary electrophoresis. Separation of fragments is dependent on their size and detection occurs by the excitation of attached fluorescent dyes by use of a laser. The laser is stationary with respect to the electrophoresis apparatus and fragments are

recorded as they pass a single point. The sequence is recorded directly onto a computer and is interpreted by software into a DNA sequence.

Sequences are then aligned. Alignment can be performed by one or more approaches: (i) on the basis of secondary structure and functional domains (ii) using one of a range of pairwise alignment of bases programs with various weighting options and gap penalties, e.g. Clustal X (Thompson *et al.* (1994)). Pairwise alignments seek to align two entire DNA regions, using a balance between matches and gaps. Every nucleotide in one sequence is compared with all in the other. The introduction of gaps is necessary to account for insertion/deletion events, but because any two random sequences could be aligned perfectly if enough gaps were introduced, gaps must be penalised. The final consensus sequence is that which has the most matches and least gaps (iii) by eye, often in relation to previously aligned sequences.

3.1.8 Phylogenetic Analysis

To examine the relationships between the DNA sequences phylogenetic trees are constructed. There are many different methods of constructing these trees and no one method is universally accepted. Methods can be divided into three groups. Distance methods are based upon the pairwise differences between the sequences. Parsimony methods search possible trees to identify those that would have the minimum number of mutational steps to account for the differences between sequences. Finally, maximum likelihood methods identify a tree that maximises the probability of obtaining the sequences. Parsimony and maximum likelihood methods are computationally intensive. Distance methods, for instance neighbour-joining, are more rapid. Neighbour-joining trees group the most closely related sequences. The length of the branches indicates the level of genetic difference between sequences.
The topology of the trees produced can be tested for reproducibility by using bootstrapping methods. This method involves generating artificial sequences by randomly sampling sites from the original data set (with replacement) and producing a sequence of equal length to the original. A tree is constructed from this new alignment. This is repeated around 100-1000 times. Comparisons are made between these and the original tree and a measure of confidence (%) is placed on each of the tree nodes (reviewed by Felsenstein 1985).

3.2 *Nuclear and mitochondrial DNA*

There are two types of DNA that an entomologist can utilise – nuclear and mitochondrial. The differences between these two types allow entomologists to use them in different situations.

Mitochondria are cellular organelles for the production of energy. Like the nucleus, mitochondria contain DNA. In theory mitochondrial DNA (mtDNA) is haploid, there is only one genome per organism, whereas nuclear DNA (nuDNA) is diploid with two copies. One genome is paternally inherited and one maternally compared with mtDNA that is only maternally inherited. However, it should be noted that heteroplasmy (the presence of more than one type of mtDNA genome) occurs widely (Rand and Harrison 1989). This heteroplasmy can be due to paternal transmission of mtDNA (Kondo *et al.* 1990). It was thought that no recombination occurred in mtDNA making mtDNA a particularly useful tool in phylogenetic studies to look at common descent (Hillis and Moritz 1990). However, recombination has been observed in some species (Ballard and Dean 2001).

The total amount of genetic information in a mtDNA molecule (15-20kb) is less than that of nuDNA (3 million kb in animals) (Avise 1994). Thus nuDNA has the potential to be more discriminating than mtDNA. There is an increased chance of locating regions of variability between insects if the nuclear genome is used.

There are only two copies of each nuDNA molecule per cell compared with hundreds or thousands of copies of mtDNA molecules (Benecke and Wells 2001). Brown *et al.* (1979 cited in Stevens and Wall 1997), estimated that the total amount of mtDNA in a cell was 5-10 times greater than that of nuDNA. The high copy number means that mtDNA is often employed when dealing with decomposed or old tissue samples. According to Malgorn and Coquoz (1999), mtDNA is the preferred choice when dealing with puparium or small parts of insects.

The two molecules also differ in structure – mtDNA is circular and has little non-coding DNA. The insect mitochondrial genome has been fully sequenced in some species and is known to consist of 13 protein-coding genes required for mitochondrial function, 22 tRNA genes (leucine and serine are duplicated), two rRNA genes and a non-coding region that contains the origin of replication (Clary and Wolstenholme 1985) (Figure 3.1). With the exception of the tRNA genes the gene order appears

highly conserved within Insecta (Wolstenholme 1992).

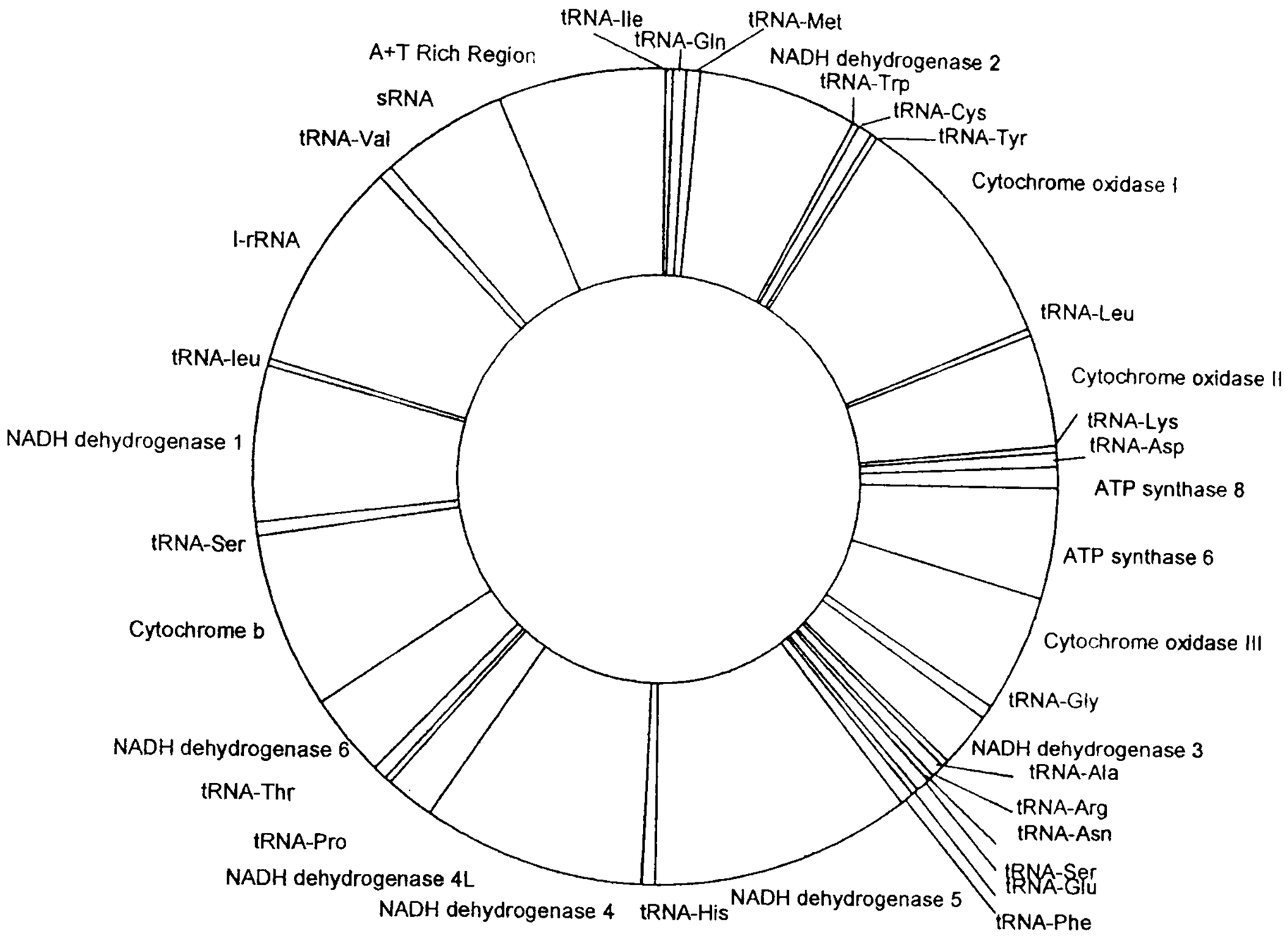


Figure 3.1. The location of genes in the mitochondrial genome of dipteran species. Figure drawn from gene boundaries given by Clary and Wolstenholme (1985) for *Drosophila yakuba*.

Conversely, nuclear DNA is linear in structure and has sections of coding DNA (exons) interspersed with non-coding lengths (introns). Non-coding DNA in the genome contains a greater amount of variation.

3.3 Variation

Variation is defined as the difference in a character between individuals. Variation provides the raw material for evolution to occur and is caused by changes, or mutations, to the DNA sequence of an individual. As DNA replicates, any errors in the bases included will produce mutations. DNA repair enzymes and proofreading enzymes present limit the number of incorrect nucleotides incorporated but some will still be included. Various environmental factors such as UV light and certain chemicals also produce mutations. Most mutations are base substitutions within the

DNA sequence. These can be transitions (purine to purine; pyrimidine to pyrimidine) or transversions (purine to pyrimidine and vice versa). Due to the degeneracy of the genetic code, changes in DNA sequence do not always affect the amino acid sequence. These mutations are known as synonymous whilst those that change the amino acid sequence are non-synonymous. The other types of mutation are insertions or deletions (indels) of bases. In coding DNA these are usually lethal mutations, as they will cause a frame-shift; all the codon positions shift causing many of the amino acids to change, thus creating a difference in protein.

Nuclear and mitochondrial genes and inter-gene regions accumulate mutations at different rates. Generally introns and non-coding regions exhibit a high mutational rate compared with the coding genes which have specific translational products that have evolved in accordance to function. Homologous genes that vary in sequence are known as alleles.

Although the mitochondrial genome contains little non coding DNA, it has previously been commented on that mtDNA as a whole accumulate mutations faster than nuDNA (Simon *et al.* 1994). This is probably due to the mitochondria having fewer DNA repair and replication proofreading enzymes. However Zhang and Hewitt (1997b) determined that this is not always true for all species, including some insects.

It is this variation that will provide the means of distinguishing one species from another (interspecific variation) and to identify different populations of species (intraspecific variation).

3.4 Identification of Geographic Populations

A population is a subset of individuals of one species that occupies a particular geographic area. This can be due to geographic obstacles not allowing random dispersal and therefore naturally creating aggregations of individuals. It can also be simply due to isolation from the broader population by distance.

Large aggregations of blowflies could be temporary, a large carcass for instance can draw blowflies from a wide area (MacLeod and Donnelly 1962). However, these researchers postulate that populations of blowflies exist regardless of the 'draw' of carrion. Aggregations exist due to positive habitat stimuli e.g. vegetation type, microclimate. Whilst some individuals will move away, other passing individuals will

be attracted to these stimuli and thus keep the aggregation of blowflies in a particular area.

To appreciate how far populations would need to be apart for them to be geographically isolated, knowledge of blowfly dispersal is required.

3.4.1 Calliphoridae Adult Dispersal

Some insects have strong dispersal abilities either by natural movements or artificially by human activities.

3.4.1.1 Calliphoridae Activity

As might be expected for poikilothermic organisms, activity is governed by temperature.

Calliphorid flies are more active during the summer than the winter months. It has been observed that during cooler months *C. vicina* adults are active during one part of the day whereas in the summer they are active twice, resting during the hottest and brightest part of the day (Norris 1965). Adams and Hall (2003) showed that in London when the temperature fell below 6°C no *C. vicina* were caught on sticky traps, although in Winter, if the weather was sunny, *C. vicina* were observed flying.

Blowfly activity is often driven by the need to feed or mate. Smith and Wall (1998) note that gravid females and protein deprived non-gravid females will be highly responsive to the stimuli of a fresh corpse. Similarly, blowflies that have just ingested nutrients are less active than those deprived of them.

3.4.1.2 Direction

The direction of flight will primarily be dictated by any olfactory stimuli attracting the blowflies. In the absence of such stimuli, Braack and Retief (1986) found that it was not the geology of an area that influenced the direction of flight of *Chrysomya* species but the vegetation. MacLeod and Donnelly (1958) monitored an artificial release of blowflies and observed their dispersal paths. They noted that the blowflies dispersed fairly randomly irrespective of habitat. The researchers concluded that if this was typical behaviour of flies in the wild that there would be a random spread of flies over the country. Conversely, their earlier work (MacLeod and Donnelly 1957) found that the natural blowfly population of the UK existed in patches. They concluded that, whilst the flight paths of blowflies are essentially random in direction, once arriving at

favourable habitat an individual would remain thus causing aggregations of blowflies across the country.

3.4.1.3 Distance

Mayer and Atzeni (1993) indicated that dispersal distances of *C. hominivorax* were influenced by habitat conditions. They used previous research to establish daily dispersal distances of 2.85km and 1.25km in ideal conditions for females and males (respectively) and 22.07km and 9.64km in less favourable conditions (i.e. depleted food sources) for females and males (respectively). Females will disperse further than males, driven by the need to find protein sources for oviposition.

Braack and Retief (1986) conducted a dispersal experiment of *Ch. albiceps* and *Chrysomya marginalis* (Wiedemann) that indicated a minimum rate of movement of 2.20km per day for *Ch. albiceps* and 2.35km per day for *Ch. marginalis.*

Lempke (1962 in Braack and Retief 1986) postulated that *C vicina* could cover 70km in one flight as specimens were found off the Belgian coast on a lighthouse. This is however just an observation by the author; the blowflies were not released in a controlled study from the mainland. Presence of the flies could be by artificial means e.g. association with man or boats.

Smith and Wall (1998) dusted *L. sericata* adults with fluorescent powder, released and captured them in baited traps. They found a mean dispersal of 135m per day. They note that if a fly has a 3-6 day lifespan in the wild (Wall 1993) it could be postulated that the blowfly would disperse an average distance of 800m per lifetime. If it is then considered that this species goes through three to four generations a year, this allows movement of approximately three km a year. They note that if maximum values of dispersal and longevity are used, a value of ten times this figure (31-42km a year) is obtained.

It is possible that these measurements of this type are biased due to wind movements and speeds but nevertheless it appears that blowflies are capable of sustained flight over relatively long distances.

3.4.1.4 Obstacles

MacLeod and Donnelly (1960) conducted a release and trap study over water and through woodland. *Calliphora vicina* were caught on the opposite side of a 200yrd (182.88m) river from the release point. They were also caught at the top of a steep

500ft (152.4m) hill face after release at the base (MacLeod and Donnelly 1958). *Calliphora vicina, C. vomitoria, L. sericata* and *Lucilia caesar* (Linnaeus) adults released on one side of a 50yrd (45.72m) strip of woodland were later caught on the other side. It appears that geographic obstacles to not hinder the flight of calliphorid flies.

3.4.1.5 Artificial Transportation

The close association with man makes it possible for blowfly species to be mechanically transported distances as opposed to flying. Evidence of this is indicated by the worldwide distribution of certain blowflies. For example, in the 1970s the sheep blowfly *L. cuprina* was transported to New Zealand from Australia and has since moved from the North to the South Island (Gleeson *et al.* 1994).

As mentioned previously, fishermen use *C. vomitoria* larvae as bait and consequently they are deliberately transported.

3.4.2 Intraspecific variation

Populations will generally exhibit a degree of genetic variation from each other. This is mainly due to non-random mating, as mates are rarely drawn from across the whole range of individuals within a species i.e. worldwide or even countrywide (Avise 1994). Most sexually reproducing species locate a partner within a localised region. This leads to local inbreeding and consequently genetic variation between populations. This intraspecific variation may allow differentiation of forensically interesting blowfly populations.

Dispersal (or migration) between the populations is of course possible, thus lowering the variation between populations. The Island model of migration assumes migrations can occur between any of the populations (MacArthur and Wilson 1967). The alternative to this model is the stepping stone model that assumes migration will only occur between populations that are close in geographic distance, thus geographic distance will be positively correlated with genetic variation between populations.

3.4.2.1 UK *Calliphora* population variation

The previous sections indicated that Calliphoridae are able to cover relatively long distances in their lifespans. Plus, *Calliphora* species go through more than one generation a year so they could potentially travel the whole country within the time a

mutation would become fixed in a population. The genome of these flies will contain mutations that appeared millions of years ago and it could be assumed that there has been some migration since then. If this is the case the genetics of UK blowfly should be panmitic (no variation).

This potential dispersal however depends upon whether blowflies need to move habitats– it has already been demonstrated that the difference in dispersal distance under favourable conditions is much lower than unfavourable conditions. England and Wales contain a mix of urban and rural areas; presumably both species do not have to move far to locate an appropriate habitat. In the case of *C. vicina,* many other synanthropic species exist e.g. rats, so plenty of other carcasses will be present within an urban environment as food/oviposition sources. For *C. vomitoria*, the rural habitat will contain other food/oviposition sources, both natural and domestic (cattle and sheep). Therefore some variation between populations might be expected, as there will not be widespread dispersal.

3.5 Part I Rationale

This aim of the first part of this research was to locate molecular markers that can be used to differentiate between *C. vicina* and *C. vomitoria* populations within England.

Initially, the developmental timings of two *C. vicina* populations were compared to assess whether populations differ in thermobiology. Then, methodology for DNA extraction was established for a range of specimen types. This is followed by the results of experimentation with potential molecular markers to distinguish between species and populations.

Chapter 4

Comparison of the development of two *Calliphora vicina* populations

It has previously been established that the duration of most developmental stages in the congeneric species *C. vicina* and *C. vomitoria* are significantly different (Greenberg 1991, Greenberg and Tantawi 1993, Ames and Turner 2003). Ames and Turner (2003) indicated that the timing of all immature stages showed highly significant differences between the two species apart from the ADH required to complete the second larval stage. The total ADH required to complete development and emerge as an adult fly was greater in *C. vomitoria* (11452) than *C. vicina* (10769). This concurs with in the experimental work of Greenberg (1991) and Greenberg and Tantawi (1993).

Other related species also have very different timings. The developmental data for the blowflies *L. sericata* and *Lucilia cuprina* (≡ *Phaenicia pallescens*) Shannon were compared by Ash and Greenberg (1975). They noted that *L. cuprina* develops faster than *L. sericata* and the rate is less variable over a range of temperatures than *L. sericata*. The authors concluded that this difference was linked to the distribution of the two species within the USA. *Lucilia cuprina*, which has a less variable development rate, has a narrower habitat range compared to *L. sericata*, which is widespread across the USA.

Lefebvre and Pasquerault (2004) noted differences between congeneric species of the 'dump' flies *Ophyra aenescens* (Wiedemann) and *Ophyra capensis* (Wiedemann).

It therefore appears that even closely related species will have significantly different developmental rates, especially if their habitat also varies.

There is a paucity of intraspecific comparisons of developmental timings in the literature. Within-species comparisons can be made upon experimental data from various authors. However, it is often difficult to form worthwhile conclusions in this way as although ADD/ADH values can be calculated, to alleviate the problem of experiments by different researchers being carried out over a variety of temperatures, definitive values for the minimum developmental thresholds have not been established for most species/populations.

Indeed, Grassberger and Reiter (2002) not only noted the differences in the ADD values of *P. terraenovae* from different researchers' work but there was also a difference in calculated minimum developmental thresholds. This variation (both ADD values and minimum developmental thresholds) is accounted for as being due to differences between geographic populations although the authors do concede they could also be due to intrinsic factors within the experimental design. These factors not only include a variety of rearing temperatures as mentioned but also food substrates on which the larvae develop. As discussed in Chapter 1, Kaneshrajah and Turner (2004) and Clark *et al.* (2005) both note differences in developmental rate correlated with tissue on which the insects were fed.

ADD values quoted in the literature should only be used if details of the minimum developmental threshold utilised in the calculations are also given, otherwise the ADD calculations will be incomparable.

Hwang (2004) compared the ADD for adult emergence for two London *C. vicina* populations in one experiment. Central London (Waterloo) and Greater London (Box Hill) were chosen as examples of urban and rural populations. Hwang postulated that the urban heat island effect of central London, which raises the temperature by approximately 2.2°C in summer, might be reflected in the blowfly developmental data from these areas. However, ADD to adult emergence for these two populations showed no significant differences, but the author did note that the variation within population replicates may have been 'masking' true between-population variation within the statistical analysis.

Hwang found no significant differences between populations and ADD to adult emergence for four different temperatures (16, 20, 24 and 28°C). This suggests that for these populations and for these temperatures, there is no relationship between genotype and environment.

Hwang concluded that the cosmopolitan nature of *C. vicina* – its presence in many types of habitat (the author found it at all trap sites in the London area, both rural and urban) and its synanthropic distribution worldwide indicates a very adaptable species. *Calliphora vicina* is a temperate species and therefore is subject to a wide range of temperatures within its lifespan. As noted for *L. sericata* (Ash and Greenberg 1975),

these types of insects are often selected for versatility rather than being perfectly adapted to specific habitats.

Wallman and Donnellan (2001) postulated whether methods to distinguish between populations of the same species were necessary as it was still unclear whether they differed sufficiently in their thermobiology. Saunders and Hayward (1998) however found that intraspecific differences did exist when examining the diapause incidence of *C. vicina* from three geographically distant areas. As mentioned previously, diapause is a strategy utilised by invertebrates to survive cold temperatures. For *C.vicina* diapause is induced by maternal experience of short daylength. Three *C.vicina* populations (Finland, Scotland and Italy) showed different survival abilities at cold temperatures. The Finnish and Scottish diapausing and non-diapausing populations were significantly more tolerant of the cold than the Italian blowflies, which showed little tolerance to the cold temperatures (0, -4 and -8°C). This work therefore indicates intraspecific differences in *C. vicina* lifecycles. It could be postulated that intraspecific variation is noted in insects from differing countries, as they are geographically distant from each other. Hwang (2004) may not have observed significant variation between the *C. vicina* Waterloo and Box Hill populations as these are only 32km apart. Movement between the two areas is theoretically possible.

This section of research therefore aimed to establish whether there is any difference in the developmental life cycles of more distant populations of *C. vicina*. The work began by comparison of a population of *C. vicina* from Cheltenham, West England with a population from London, East England.

4.1 Materials and Methodology

4.1.1 Samples

Adult *C. vicina* were caught using the funnel trap in Cheltenham (see Figure 2.1 for location). Any eggs oviposited on bait were placed onto fresh pig liver in containers (Figure 2.6) at 20°C. Emerging adults were added to laboratory populations.

4.1.2 Experimental Sample Collection

Fresh pig liver (approximately 25g) was presented to the cage when eggs were required and the time of oviposition noted as time 'zero'.

Pig liver was chosen following Byrd and Butler (1997) who presented a variety of meats to *C. rufifaces* larvae of which 75-80% fed upon pig meat over beef, chicken and bovine blood. Pig liver had also been used previously in this laboratory (Ames and Turner 2003, Hwang 2004). The first egg batches of oviposited were discarded, especially if oviposition had not occurred for a few days, to avoid potential precocious larvae. Liver was presented at times to ensure metamorphosis events within observation periods (0800 hours to 1800 hours daily). Collection of eggs was done on three separate occasions. Liver was presented to females for only a short period so that eggs deposited would all be of very similar age (within half an hour). Approximately 30-45 eggs were used in each replicate. Liver was then placed in containers as described in Section 2.3 (Figure 2.6) and immediately placed at 20°C in a cooled incubator (LMS, Sevenoaks, Kent UK). Liver was added regularly so that it was always in excess to avoid competition and increased temperatures from larval masses (Byrd and Butler 1997).

4.1.3 Observation of Development

Replicates remained at 20°C for the whole lifecycle until adult emergence. Initially, eggs were viewed half hourly until all had hatched and hourly thereafter (between 0800 and 1800 hours daily). Larval stages were examined under a dissection microscope to determine developmental stage. To differentiate between stages the posterior spiracular slots were counted. As larvae morph from one stage to the next they gain a pair of slots in the posterior spiracles until the third stage when they appear as Figure 2.9.

For this experiment, transition to the pupal stage was defined as the time of formation of the white puparium and later, as adults emerged, they were counted and removed from the containers. The time (hours since oviposition) when subjects progressed from stage to stage was recorded. The laboratory environment (where observations occurred) was a constant temperature (20°C ±1°C) so that during observation insects were not exposed to variable temperatures.

4.1.4 Calculation of Accumulated Degree Hours (ADH)

ADH was calculated according to the following formula

ADH= Developmental time (h) x Temperature (°C)

For comparison with the 'control' results in Ames and Turner (2003) no correction was applied to account for a lower developmental threshold temperature.

4.1.5 Statistics

Statistical analyses of results were computed with the use of MINITAB software. One-way Analysis of Variance (ANOVA) was conducted. This statistical test indicates any significant differences between experimental conditions by comparing the variation within the different experimental conditions with the variation between them.

4.2 Results

Temperatures were taken from the Meteorological Office website for the two weather stations nearest to the two populations (www.metoffice.com/climate/uk/stationdata) and are presented in Table 4.1. Temperatures for the year 2003 were broken down into seasons and also included.

Table 4.1. Average temperatures (°C) for the Ross on Wye (Cheltenham) and Greenwich (London) weather stations obtained from the Meteorological Office. Spring (Mar-May), Summer (Jun-Aug), Autumn (Sep-Nov) and Winter (Dec-Feb).

Time Period	Average Cheltenham Temperatures (°C)			Average London Temperatures (°C)		
	Daily Min	Daily Max	Mean	Daily Min	Daily Max	Mean
1959-2004	6.3	14.0	10.1	7.2	14.8	11.0
2003	6.5	15.3	10.9	8.1	16.5	12.3
Spring 2003	5.3	15.1	10.2	6.6	16.2	11.4
Summer 2003	12.8	22.8	17.8	14.7	24.6	19.7
Autumn 2003	6.3	15.3	10.8	8.3	16.6	12.4
Winter 2003/4	3.6	8.7	6.1	3.9	9.0	6.4

The experimental raw data are presented in Appendix I. These data were subjected to a one-way ANOVA to check whether there was any significant difference between replicates for each developmental stage (Table 4.2). Only if replicates are statistically similar can they be pooled for future statistical calculations.

Table 4.2. Mean cumulative duration of each *C. vicina* (Cheltenham) developmental stage along with between replicates 1-way ANOVA results (F–test statistic; p-probability value).

Stage	Number of insects			Mean cumulative duration of stage (h)			Between replicates ANOVA	
	1	2	3	1	2	3	F	p
Egg	37	45	49	23.9	23.3	22.9	2.47	0.089
L1	37	36	45	43.0	43.5	42.9	0.24	0.787
L2	37	36	43	88.1	89.9	90.0	1.83	0.166
L3	36	43	50	207.2	207.0	206.6	0.03	0.975
Pupal	33	35	42	542.4	538.2	537.6	2.05	0.133

There was no significant difference between the samples at the 5% significance level, thus allowing the replicates to be pooled. After replicates were pooled within each developmental stage, overall mean ADH values were calculated and compared to London *C. vicina* (data reproduced from Ames and Turner 2003). The data were subjected to a one-way ANOVA to assess whether significant variation exists between the two populations. Results are presented in Table 4.3.

Table 4.3. Mean ± standard deviation, minimum and maximum cumulative ADH of lifecycle stages for *C. vicina* London and Cheltenham populations along with probability values after ANOVA. * significant at 5% significance level.

Stage	*C. vicina* London Cumulative duration of Stage (ADH)		*C. vicina* Cheltenham Cumulative duration of Stage (ADH)		ANOVA	
	Mean	(Min-Max)	Mean	(Min-Max)	F	p
Egg	493.0±26	435-555	466.5±43	365-555	43.34	0.000*
L1	850.7±98	725-1010	862.4±88	640-1120	0.84	0.360
L2	1732.7±114	1480-2380	1787.1±99	1600-2080	14.47	0.000*
L3	4200.0±258	3580-5055	4138.2±239	3580-4540	4.11	0.044*
Pupal	10769.0±250	10020-11260	10784.0±230	10300-11260	0.23	0.629

The ANOVA indicates that there are significant differences between some of the immature stages of the two *C. vicina* populations. The ADH required to reach the first, third larval stages and pupal stages are significantly different between the populations. ADH values to reach the first and third stages are both significant at the 1% level and ADH to pupal stage is significant to the 5% level. To reach the first larval stage the Cheltenham population requires less ADH than the London population (466.5 versus 493.0 ADH). However, to reach the third larval stage the London population accumulates less degree hours (1732.7 ADH) than the Cheltenham (1787.1 ADH).

4.3 Discussion

The results of this experiment indicated that there is slight but statistically significant variability between different populations of *C. vicina*. These significant differences were limited to the cumulative ADH to reach the first, third and pupal stages. Even though the mean ADH value to reach the second larval stage is greater for Cheltenham (not significant) the minimum ADH value is actually lower for the Cheltenham population. This is important, as it is the oldest larvae that will be collected by a forensic entomologist at a scene of crime, therefore minimum ADH values should also be considered. Taking minimum values into account, it appears from these data that initially the Cheltenham population requires less ADH units to progress through the first two stages. Then the London population requires significantly less ADH to go through the final three stages, including the pupal stages which, although not significantly different, the London population has the lower minimum and mean value.

The London population is subject to the urban island heat effect. Hwang (2004) noted that this raised the temperature by approximately 2.2°C in summer. As demonstrated by the temperature values obtained from the local weather stations the average difference over a year (2003) is 1.4° C higher in London and the difference in summer is 1.9°C higher. Thus insects in London experience higher temperatures. The immature stages (egg and first larval stages) are the most vulnerable and therefore it is beneficial to limit the duration of these stages. This could be a reason for the Cheltenham population requiring less ADH to finish these stages, as they will be

subjected to slightly lower temperatures than the London population. It does not explain why the ADH for the second larval stage is significantly less for the London population.

An interesting result is that the total ADH required to transform from egg to adult is not significantly different between the two populations. This concurs with the work of Hwang (2004) where total ADD for the urban and rural London populations of *C.vicina* did not vary significantly. However Hwang did not measure ADD requirements for any of the immature stages and so it cannot be established whether significant differences were actually present between the two *C. vicina* populations for the immature stages. As mentioned before it is not always possible to rear immatures from crime scenes and therefore the data for the ADH requirements to reach all the life cycle stages for these species is very important for a forensic investigator.

Hwang (2004) noted that the total ADD was significantly different between males and females, with males requiring fewer thermal units. In this work, insects were not sexed and as cohorts were obtained from the same oviposition time, it was assumed that the ratio of males to females was about 1:1 and that this would be equal in all replicates from both populations and so the influence of sex on replicates can be discounted.

These ADH values were then compared with other values obtained from the literature for other *C. vicina* populations (Table 4.4).

Table 4.4. Developmental time (egg to adult emergence) for *C. vicina* populations from data presented in the literature. *indicates where ADH values have been calculated for this work (i.e. ADH values were not presented by the original authors).

Reference	Location	Temperature research conducted (°C)	Developmental time as presented (egg to adult)	ADH
Greenberg (1991)	Chicago, USA	19	562h	10678
Marchenko (2001)	Russia	19	22.9d	10442.4*
		20	21.6d	10368*
Kamal (1958) presented in Smith (1986)		27	18d	11664*
Kamal (1958) recalculated in Higley and Haskell (2001)		26.7	508h	13563.6*
Anderson (2000)	Vancouver, Canada	20.6	Min – 514.8h	10604.9*
Johl and Anderson (1996)	Vancouver, Canada	24	18.1d	10425.6*
Hwang (2004)	London – Waterloo	20	533.21h	10664.2*
	London – Box Hill	20	528.26h	10565.2*
Ames and Turner (2003)	London – Waterloo	20	538.5h	10769
Ames	Cheltenham	20	539.2h	10784

Table 4.4 indicates that the majority of ADH values are between 10368 and 10784 ADH. The exceptions are the data presented by Smith (1986) and Higley and Haskell (2000) as recalculations of the original work of Kamal (1958). These high values could be due to the high temperatures at which the experiments were conducted. It

could be that these temperatures are beyond the linear phase of the time/temperature relationship as mentioned in Section 1.4.6, and therefore the ADH model does not truly apply. For the experiments in this work 20°C was selected as the experimental temperature. This was primarily to allow comparison with the previous work of Ames and Turner (2003) but also as Hwang (2004) indicated that this temperature was optimum for *C. vicina* life characteristics.

Although the results (excluding the Kamal figures) are of similar magnitude, there are differences. The Russian values are lower than the rest along with the Canadian values of Johl and Anderson (1996). These insects are living within colder climes and therefore a reduction in the requirement of degree hours to develop would not prolong overall development duration.

As discussed, it should be noted that the slight differences could however be due to differences between the conduct of experiments by the researchers. As mentioned, Kaneshrajah and Turner (2004) showed that the substrate offered as a food medium in laboratory growth experiments affected the overall developmental lifecycle timings for *C. vicina*. Clark *et al.* (2005) noted a similar phenomenon for *L. sericata*. For this work, pig liver was used as food for developing larvae to allow direct comparison with the previous results of Ames and Turner (2003). Larvae taken from various sites on a corpse should be compared to empirical life cycle developmental timings based upon the same organ food substrate.

Previous experiments on developmental timings have often used larval length as an indicator of age (Byrd and Butler 1997 amongst others). Whilst larval length is a good indicator (Grassberger and Reiter 2001) and indeed is often used in crime scene specimens to establish age, it must be considered that transition from stage to stage is an actual factor of age whereas length is actually a measure of somatic growth (Dadour *et al.* 2001). As noted in Chapter 1, larval length has been shown to vary according to other factors such as competition.

This work indicates that some statistically significant differences do exist between UK *C. vicina* populations' immature developmental timings. However, it should be noted that these findings must be treated with a degree of caution. For example, whilst the difference between developmental time to the first larval stage is statistically different,

in reality they are small (approximately 1.5 hours at 20°C). This study was conducted under controlled laboratory conditions. In the environment, these small differences may not be noted due to the less controlled conditions of the field. Therefore, although these results do not show large differences between immature *C. vicina* developmental rate for the two populations in this work, it would be worthwhile examining other population development cycles to assess whether greater differences do exist.

Chapter 5

Methodologies for location of DNA molecular markers

5.1 Sample Preparation

Samples used in this work were either freshly killed by freezing or killed previously and stored in 95% v/v ethanol at -20°C. Samples that were freshly killed were washed in ddH_2O to remove any exogenous material before being placed in individual 1.5ml tubes (Eppendorf). Stored samples were defrosted at room temperature until excess ethanol had been removed before samples were placed in 1.5ml tubes.

To prevent any contamination between samples they were homogenised within separate 1.5ml tubes, rather than in a pestle and mortar. Liquid nitrogen was only added to the tubes containing adults (larvae were easier to homogenise without freezing) and a sterile autoclaved micropestle (Eppendorf) was employed to breakdown the sample tissue.

5.2 Assessment of DNA Extraction Methods

To establish which method of DNA extraction would be used in this study, three methods of DNA extraction were conducted on the thoraxes of *C. vicina* and *C.vomitoria* adult flies.

5.2.1 Phenol:Chloroform Extraction

This method was used by Sperling *et al.* (1994) for DNA extraction. Specimens were homogenised in liquid nitrogen and added to a 1.5ml tube (Eppendorf). To each tube, 400µl of Lifton buffer (0.2M Sucrose (BDH); 0.05M EDTA (Sigma); 0.5% w/v SDS (Sigma); 0.1M Tris (Sigma); pH 9.0) was added and the sample vortexed for 30s. Then, 120µl of 8M potassium acetate (Sigma) was added to samples on ice. Samples were then centrifuged at maximum speed (20,000 x g) for 10 minutes. The supernatant was removed to a fresh 1.5ml tube and an equal volume of phenol was added. Samples were inverted to mix, centrifuged and the aqueous layer extracted to a fresh 1.5ml tube. This was repeated using chloroform:isoamyl alcohol (24:1) (BDH:Sigma). An equal volume of ice-cold isopropanol (Sigma) was added to the sample and this was put on ice for 10 minutes. The samples were then centrifuged for 20 min at 20,000 x g. The supernatant was removed and discarded and the remaining

pellet was washed three times using 70% v/v ethanol (BDH). Samples were dried under vacuum and 50µl ddH_2O was added and left overnight at 4°C to allow the DNA to diffuse into solution.

5.2.2 Rapid Salt Hydrolysis DNA Extraction

This extraction protocol is based upon the work of Aljanabi and Martinez (1997). To the homogenised sample, 400µl of TEN buffer (10mM Tris-HCl, 2mM EDTA, 0.4M NaCl (BDH); pH 8.0) was added. Then 40µl of 20% w/v SDS (Sigma) and 8µl of 20mg/ml Proteinase K (Sigma) were added and mixed well. The samples were incubated at 60°C for two hours in a heating block (Anachem, UK), after which 300µl of 6M NaCl was added to each sample. Samples were vortexed for 30s at maximum speed, and tubes centrifuged for 30 min at 13,000 x g to spin down the cell debris. The supernatants were then transferred to fresh tubes. An equal volume of ice-cold isopropanol (BDH) was added to the samples, which were mixed well, and placed at –20°C for 1h. Samples were then centrifuged for 20 min, 4°C at 13,000 x g. The pellet was washed three times with 70% v/v ethanol (BDH), dried and finally resuspended in 50µl ddH_2O. The samples were left overnight at 4°C to allow the DNA to diffuse into solution.

5.2.3 Spin Column Extraction Method

The QIAamp® DNA Mini Kit (QIAGEN Ltd, UK) was used according the manufacturer's Tissue Extraction protocol. Tissue Lysis buffer ('ATL', 180µl) was added to the sample that was then homogenised. After addition of 20 µl (20mg/ml) Proteinase K, samples were thoroughly mixed and incubated at 56°C for two hours. After this, 200ml Lysis buffer ('AL') was added to each sample tube, vortexed and incubated at 70°C for ten minutes. Two hundred microlitres of 99% v/v ethanol (BDH) was then added and mixed. The contents of each tube were added to a QIAamp Spin Column. Columns were centrifuged at 6,000 x g for one minute. The filtrate was discarded and 500µl of Wash buffer 'AW1' was added and tubes centrifuged at 6,000 x g for one minute. The filtrate was again discarded and 500µl Wash buffer 'AW2' was added to each column and then centrifuged at 20,000 x g for three minutes.

The columns were transferred to clean 1.5ml Eppendorf tubes and 100µl of ddH_2O was added. Each tube was incubated at room temperature for one minute and then centrifuged at 6,000 x g for one minute to elute the DNA. The extracted DNA could be used immediately.

5.2.4 Agarose gel electrophoresis

Sample DNA (5µl) was loaded on a 1% w/v agarose gel to separate. Gels were made using agarose (Sigma) and TAE (Tris; acetic acid; EDTA; pH 8.3) solution. Electrophoresis was carried out at 95 volts until the marker dye had migrated a suitable distance down the gel (approximately 30-45 min). Gels were then stained in ethidium bromide solution (approximately 0.02% v/v). Ethidium bromide intercalates with DNA molecules and fluoresces at 545nm, thereby allowing visualisation of the DNA under UV light. A 1kb or 100bp DNA molecular ladder was also run alongside the samples. This consists of DNA fragments of known length for comparison with unknown samples. A quantitative DNA ladder includes certain length fragments of known DNA amount. The intensity in fluorescence of unknown samples can be compared either by eye or densitometry.

The gel was visualised and photographs taken and recorded using AlphaImager™ version 3.3 (Alpha Innotech Corporation, CA, USA).

5.2.5 PicoGreen Quantification

PicoGreen is a dye that selectively binds to double stranded DNA. When bound it fluoresces (at a wavelength of 535nm) on excitation at 485nm. The amount of fluorescence is proportional to the amount of DNA in the sample. A PicoGreen assay was set up according to manufacturer's instructions (Molecular Probes, Oregon, USA). DNA standards (100, 80, 60, 40, 20, 10, 5 and 2.5 ng/µl) were included on the 96-well plate (CoStar, Corning, NY, USA). Ten microlitres of each sample were added to 190µl of PicoGreen mix in wells. Each sample was duplicated on the plate. Plates were read on a Cytofluor 4000 Plate reader (PerSeptive Biosystems). Using the standards, calibration curves were created in Excel (Microsoft®). From these graphs the concentration of the samples could be established.

5.2.6 Results and Discussion

There are many methods of DNA extraction. The three in this section were chosen as they represent different types of extraction e.g. a phenol/chloroform organic method; a non-organic method and a spin column kit. All three methods will extract total DNA i.e. both nuclear and mitochondrial.

The different DNA extraction techniques produced average DNA amounts (± standard error) of 4.9μg (± 1.0), 6.1μg (± 0.2) and 6.7μg (± 1.7) for phenol/chloroform, rapid salt and spin column methods respectively. The spin column method extracted the greatest amount of DNA but the rapid salt protocol was the most consistent with the lowest standard deviation.

From this initial quantification is would appear that rapid salt hydrolysis provides a cheap technique that produces a consistently high yield of DNA from this type of sample. However the agarose gel picture of the DNA extraction samples (Figure 5.1) indicated that the rapid salt method samples contained some degraded DNA. High molecular weight DNA is present as the discrete band at the top of the gel. RNA appears as a broad band at the bottom of the gel and is evident in all samples. Degraded DNA appears as a smear between the discrete band and the RNA broad band.

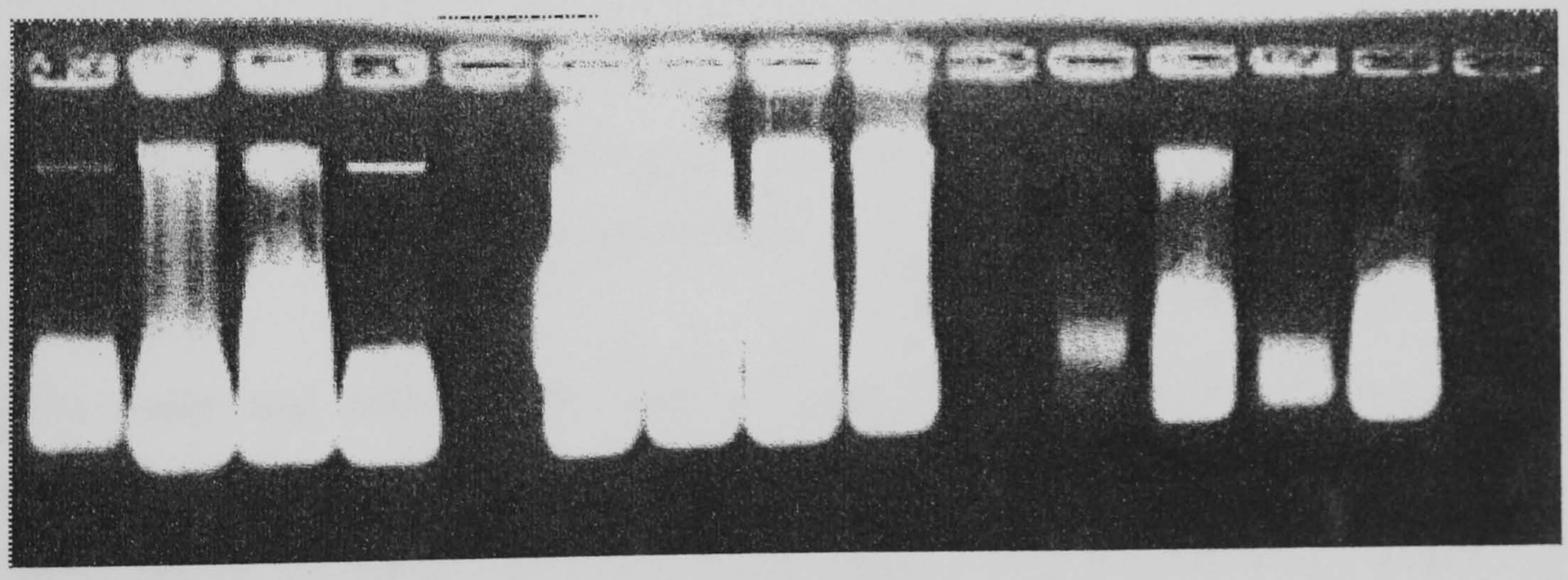


Figure 5.1. Examples of differently extracted DNA samples. Lanes 1-4 extracted using Qiagen spin columns. Lanes 6-9 extracted using rapid salt hydrolysis. Lanes 11-14 extracted using phenol/chloroform.

From these analyses it appeared that the spin column method produced the highest yield of good quality DNA. This method is more expensive per sample than the other methods but is much quicker, allowing higher throughput of samples. Spin columns

(QIAGEN Ltd) were therefore used in the majority of DNA extractions in this research.

Recently, Harvey (2005) has proposed the use of Whatman FTA™ cards for extraction. These provide certain benefits over the use of traditional DNA extraction techniques, especially the ease of room temperature space saving storage. However, as a punch of the cards is placed directly in the PCR reaction, no quantification was conducted and so these cards cannot be compared with the results of this study.

5.3 Oligonucleotide Primers

Oligonucleotide primers for use in PCR were either adapted from the literature or were designed using Primer 3 online software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). This software designs primers to produce amplicons of designated lengths after user input of a template sequence. Primers are designed not to form primer-dimers and should have equivalent melting temperatures.

Once designed, primers were either manufactured by Invitrogen (UK) or by Biomolecular Service, King's College, London.

5.4 Amplification (PCR)

PCR reactions were carried out using a total reaction volume of 25µl in a 0.2ml thin walled PCR tube (Ambion). The majority of reactions consisted of 0.5µl of each primer (10µM) along with 2µl template DNA (30-60ng DNA). Malgorn and Coquoz (1999) found that 10-100ng of insect DNA gave the best results in subsequent post-PCR analysis.

Sterile water was added to a volume of 12.5µl. To reduce the generation of nonspecific products and primer-dimer artefacts, the samples were heated for 1 minute at 94°C before 12.5µl of Readymix™ Red Taq™ PCR Reaction Mix (Sigma, UK) was added. This product contains 0.75 Units of *Taq* DNA polymerase, 1.5mM $MgCl_2$, 0.2mM dNTPs and buffer (10mM Tris-HCl, 50mM KCl) in the final volume. Samples were then loaded onto the GeneAmp® PCR System 9700 Thermal Cycler (PE Applied Biosystems, USA). Typically the following cycles were run;

Number of Cycles	Temperature (°C)	Duration	Cycling Step
1	94	2 min	Hot Start *Taq* polymerase
30-35	94	30 s	Denaturation
	Dependent on primer pair	30 s	Annealing of primers
	72	90 s	Extension of DNA strand
1	72	10 min	Completion of DNA strand

The final cycle is to allow complete non-template adenylation of all amplicons.

If required, PCR products were separated by agarose gel electrophoresis as described in Section 5.2.4. Loading buffer is not required as the Red Taq™ contains a dye.

5.5 Purification

Amplicons were purified to remove residual reagents from the PCR, such as dNTPs, by using GFX™ PCR and Gel Band Purification spin column kit (Amersham Biosciences, UK). The kit was used according to the manufacturer's instructions. PCR products were mixed with buffer provided and deposited onto the spin columns. After a series of wash stages, DNA was eluted off the columns with an appropriate amount of ddH_2O (usually 20-50µl). The volume of ddH_2O depended upon the final concentration of the DNA sample required.

If DNA needed to be purified from agarose gel bands, then the appropriate gel band was excised using a scalpel and placed in buffer (provided with the Amersham kit) within a 1.5ml tube. Tubes were placed at 60°C for 5-15 min until the gel had dissolved. The liquid was then transferred to a spin column and the remainder of the protocol was as above.

5.6 Sequencing

Sequencing of the purified products was carried out on a 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

5.6.1 Cycle Sequencing

To prepare dye terminated DNA fragments the template was primer extended as follows. The samples were mixed with sterile water to a final concentration of 1ng/µl

per 100 basepairs of fragment to be sequenced. Therefore for a 500bp fragment this gives a final DNA concentration of 5ng/µl. Two reactions were set up in separate 0.2ml thin walled tubes, one using 2µl forward primer (5µM) and one with 2µl of the reverse primer (5µM) along with 4µl of the diluted purified PCR product and 4µl of Big Dye™ Terminator Cycle Sequencing v3.1 Reaction Mix (PE Applied Biosystems, USA). The samples were vortexed and centrifuged briefly. They were then run on the following thermal cycle – [96°C for 10s; 50°C for 5s; 60°C for 4 min] for 25 cycles as stated in the manufacturer's protocol (Applied Biosystems).

5.6.2 Purification

The labelled DNA fragments were then purified. The 10µl sample was added to 26µl of ethanol:1M sodium acetate pH 5.2 (25:1) and 5µl EDTA (25mM) in a 0.5ml Genetic Analyser sample tube. This was incubated at room temperature for 10 min before being spun at 14,000 x g for 10 min. The supernatant was removed and discarded and the pellet washed with 70% v/v ethanol and vacuum dried.

Template Suppression Reagent (15 µl) (PE Applied Biosystems, USA) was added and the sample placed into the 310 Genetic Analyser (Applied Biosystems) for sequencing, according to the manufacturer's protocol.

5.7 Sequence Analysis

Sequences were analysed using BioEdit software (Hall 1999) and multiple alignments created using CLUSTAL X (Thompson *et al.* 1997). Forward and reverse sequences from the same sample were compared, any discrepancies resolved by referring to original sequence chromatograms and a consensus sequence for a particular sample was established. Non-included dye-labelled nucleotides and primers caused broad peaks masking the initial bases in both the forward and reverse sequences of some samples. Therefore these regions were not included in the analysis.

Sequences produced were compared to those already in the National Centre for Biotechnology Information (NCBI) database of sequences (GenBank) by use of the online Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast). This tool allows rapid sequence comparison (Altschul *et al.* 1990). BLAST searches provide an Expect Value (E) as a measure of the similarity between the query sequence and a GenBank sequence. The query and database sequence match is

assigned a score and the expect values are the number of sequences expected by chance, given that particular score, within the database the size of GenBank. The expect values decrease exponentially with the score. The longer, more complex the sequence is, in theory, the lower the chance of an exact match being 'hit' by chance.

Analyses of molecular variance (AMOVA) were conducted to assess whether variation between populations was significantly different to the variation within populations. This analysis is similar to the conventional ANOVA but specifically utilises genetic distances between sample groups. This was done using Microsoft® Excel and the linked GenAlEx software (Peakall and Smouse 2005). Populations containing only one sample had to be removed before calculation of AMOVA as there is no within population variance value for these populations. This calculation provides a Φpt statistic for haploid data analogous to Wright's F-statistics.

Differences between sequences were assessed using the MEGA v.3 software package (Kumar, Tamura and Nei 2004). These differences are a measure of the nucleotide substitutions between two sequences and included the Tamura-Nei correction factor. This corrects for whether the nucleotide substitution is a transversion or transition and also whether it involves pyrimidines or purines. The values calculated are known as the genetic distance between sequences. Neighbour-joining trees (with 1000 bootstrapping replicates) can then be constructed from the pairwise distances established. The branch lengths separating the samples are equivalent to the genetic distances between the nucleotide sequences. Neighbour-joining trees were constructed from the pairwise distances established using MEGA software. The genetic distances between populations can be compared with geographic distances graphically in Excel and also statistically using the Mantel test for matrix correspondence in GenAlEx (999 random permutations). This test provides a correlation coefficient (R_{xy}) between the two types of distance between populations.

Once the methodologies for DNA analysis had been established, potential molecular markers were examined for their suitability.

Chapter 6

Potential Molecular Marker - Cytochrome oxidase I

Cytochrome oxidase (CO) is a respiratory enzyme which is encoded by genes located in both the nuclear and the mitochondrial genome. CO consists of three mitochondrially encoded subunits and at least four subunits encoded by the nuclear genome (Smith *et al.* 1996). The mitochondrial cytochrome oxidase subunit I (COI) amino acid sequence is the largest of the subunits. Clary and Wolstenholme (1985) found that in *Drosophila yakuba* Burla COI was composed of 511 amino acids, cytochrome oxidase subunit II (COII) 228 amino acids and cytochrome oxidase subunit III (COIII) 261 amino acids. COI spans the membrane and is involved in electron transport and proton translocation across the membrane. Lunt *et al.* (1996) compared the amino acid sequence conservation in different Insecta groups and deduced the location of 25 different structural regions of the COI protein. These regions consist of 12 transmembrane helices (M1-M12) along with six external loops (E1-E6), five internal loops (I1-I5), an internal carboxyl (COOH) terminal and an internal amino (NH_2) terminal (Figure 6.1). The different structural regions have different DNA sequence variability – the COOH region is the most variable, with E1, M3, E2, I2, I4, M9 and M12 being the next variable regions (Lunt *et al.* 1996). Regions of high variability can be used to distinguish closely related species whereas more conserved regions can differentiate more diverse species, without the interference of possible intraspecific variation. This difference in variability across the gene has made COI popular for phylogenetic studies.

The fact that areas of variability are flanked by cross species conserved regions makes it possible to design primers complementing these so that the variable areas can be amplified in all fly species. After Lunt *et al.* (1996), Zhang and Hewitt (1997a) designed 10 conserved Insecta COI primers to complement those of Simon *et al.* (1994) and Kambhampati and Smith (1995).

Researchers have used the CO genes to distinguish between insects of forensic significance. Wallman and Donnellan (2001) have demonstrated the utility of COI and COII gene sequences for the identification of forensically important species of

blowflies from South-Eastern Australia. The value of CO was also confirmed by a recent study in Western Australia using a 278bp region of the COI gene (Harvey *et al.* 2003). Harvey *et al.* identified 45 regions that distinguished three genera – *Lucilia, Calliphora* and *Chrysomya.* Of these 45 regions, only two areas are actually different for all three genera, the rest distinguish one from the other two. Harvey *et al.* compared two species of C*alliphora* (*Calliphora augur* (Fabricius) and *Calliphora dubia* (Macquart)) and located three areas in the part of the COI gene they examined that showed variation between these species (Harvey *et al.* 2003).

Malgorn and Coquoz (1999) found COI sequence differences of 1.97-8.22% between combinations of *Lucilia ampullacea* Villeneuve, *L. caesar, L. illustris* and *L. sericata.* The low level of 1.97% was between *L. caesar* and *L. illustris*, which are closely related and morphologically very similar.

Wells *et al.* (2001b) used a region of the COI gene not only as a method for forensic identification of Sarcophagidae but also to reveal information on the phylogeny of these species and aid in greater understanding of their biology (morphological similarities).

Otranto *et al.* (2003) demonstrated the wide variation between Oestridae species using a region of the COI gene. This variation ranged from 27% for two species from different subfamilies to 0.7% for two closely related species within a subfamily.

Schroeder *et al.* (2003) examined a COI region of German *C. vicina* and *C. vomitoria* (similar to the UK, these are two of the more common blowfly). The region was chosen after Sperling *et al.* (1994) and is in the 3' half of the COI DNA sequence. They indicated that PCR-RFLP could be used to distinguish German *C. vicina, C.vomitoria* and *L. sericata.* The most recent study using COI has examined the Calliphoridae and Sarcophagidae in Japan (Saigusa *et al.* 2005) providing the first COI sequence for *Calliphora lata* Coquillett.

Whilst COII has been used as a potential molecular marker for Calliphoridae (Smith *et al.* 1996), it was decided in this study to concentrate on COI. Wallman and Donnellan (2001) demonstrated that COII could not differentiate between sister species *C. augur/C. dubia* and *Calliphora albifrontalis* (Malloch)*/Calliphora stygia* (Fabricius), consequently it was decided to avoid this gene for *C. vicina* and *C.vomitoria.*

After careful consideration of all the studies so far undertaken, the region selected for this investigation is at the NH_2 end of the gene product. Primers from Simon *et al.* (1994) were chosen to amplify this region. They are of similar location within the gene region as the UEA3/UEA4 primer combination of Zhang and Hewitt (1997a). According to Zhang and Hewitt (1997a) the amplicon amplified using this primer pair covers one of the two most variable regions of the COI gene and therefore will be useful for distinguishing closely related species and population studies. Funk. (1999) used this region on beetles and found it provided enough variation for inter and intra specific analysis.

In relation to the protein structure (Figure 6.1) the region examined in this work corresponds with I1-E3.

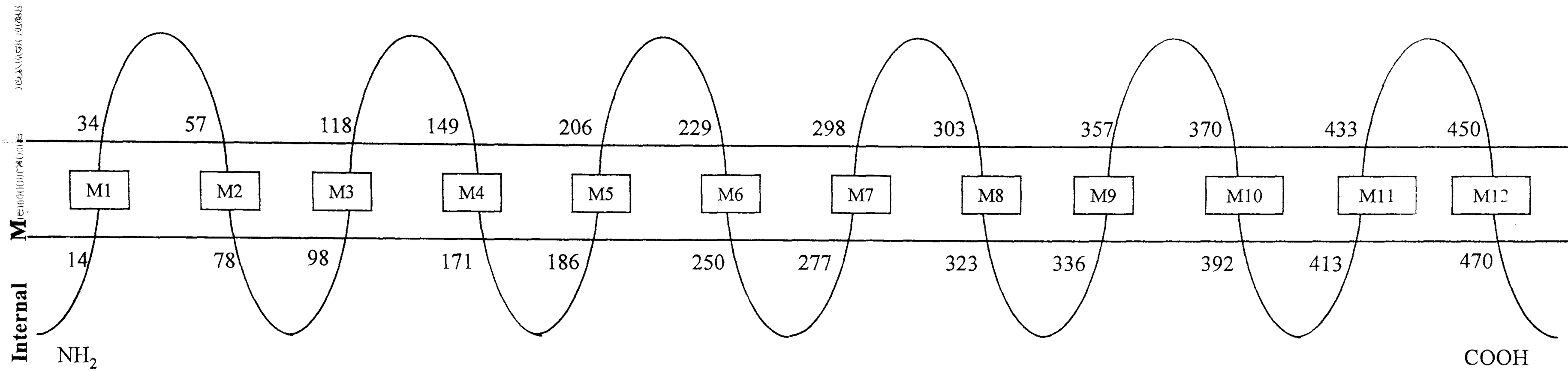


Figure 6.1. 2D structure of membrane spanning protein cytochrome oxidase subunit I (COI). Numbers represent amino acid residues located either side of the membrane of the general insect COI amino acid sequence (based upon sequences and adapted from structure presented in Lunt *et al.* (1996)).

6.1 Materials and Methods

6.1.1 Samples/Extraction Method

Samples of *C. vicina* and *C. vomitoria* were taken from laboratory populations, a commercial supplier and from those specimens caught in the wild. Both adult flies and third stage (wandering) larvae were included in this study.

Samples were subjected to preparation and DNA extraction using spin columns as discussed in Section 5.1 and 5.2.

6.1.2 DNA Amplification (PCR)

Thermal cycling was conducted using the extracted DNA as a template along with primers from Simon *et al.* (1994) (Table 6.1) as in Section 5.4.

Table 6.1. Primers for amplification of a partial mitochondrial Cytochrome oxidase I region from Simon *et al.* (1994).

Primer Name	Sequence of Primer
C1 - J- 1718	5' GGAGGATTTGGAAATTGATTAGTTCC 3'
C1 - N - 2191	5' CCCGGTAAAATTAAAATATAAACTTC 3'

Samples were then loaded onto the GeneAmp® PCR System 9700 Thermal Cycler (PE Applied Biosystems, USA). The following cycles were run: 94°C for 2 min; 30 cycles of [94°C for 30s; 60°C for 30s; 72°C for 60s]; 72°C for 15 min; 4°C to finish.

Next, 5µl of PCR product was run on a 1% TAE agarose gel containing ethidium bromide to check for amplified products (as in Section 5.2.4). A 1kb DNA ladder was run along with the products to provide an estimation of product size.

6.1.3 Purification

PCR products were purified using GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, UK) as in Section 5.5. DNA was eluted from spin columns with 30µl ddH_2O.

6.1.4 Sequencing

Sequencing of the purified products was carried out on a 310 Genetic Analyser as in Section 5.6.

6.1.5 Comparison of Sequences

Sequences were analysed using BioEdit software as in Section 5.7. After all the sequences were aligned consensus sequences for both species were produced.

6.1.6 Restriction Enzyme Analysis

Restriction maps were created based upon the consensus sequences using BioEdit software (Hall 1999). Each enzyme was assessed as to whether different fragment sizes for each species would be produced. After this analysis the restriction enzyme *Sfc*I was chosen and used according to manufacturer's instructions (New England Biolabs, USA).

Each reaction mix consisted of 0.5µl of *Sfc*I (1U), 2µl of 10X NEB Buffer 4 (50mM potassium acetate; 20mM tris-acetate; 10mM magnesium acetate; 1mM DTT), 0.2µl 100X BSA, approximately 1µg DNA and ddH_2O to make the total volume to 20µl. Reactions were incubated for 1 hour at 37°C.

Samples (5µl) were then subjected to electrophoresis on a 1% TAE agarose gel and visualised after staining with ethidium bromide.

6.1.7 Phylogenetic Analysis

Neighbour-joining phylograms were constructed from two datasets. The first being the sequences established in this work and the second from an alignment established from all the Calliphoridae samples in GenBank for the equivalent COI region. Bootstrapping of 1000 replicates was carried out for each tree.

6.2 Results

6.2.1 Interspecific variation

Figure 6.2 illustrates a selection of purified amplicons of the partial COI gene region produced with these primers. The amplification produced one clear band on the gel, which allowed products to be sequenced straight after PCR purification. Comparison with the DNA size ladder indicates the products are above 500 base pairs in size for both *C. vicina* and *C. vomitoria* species.

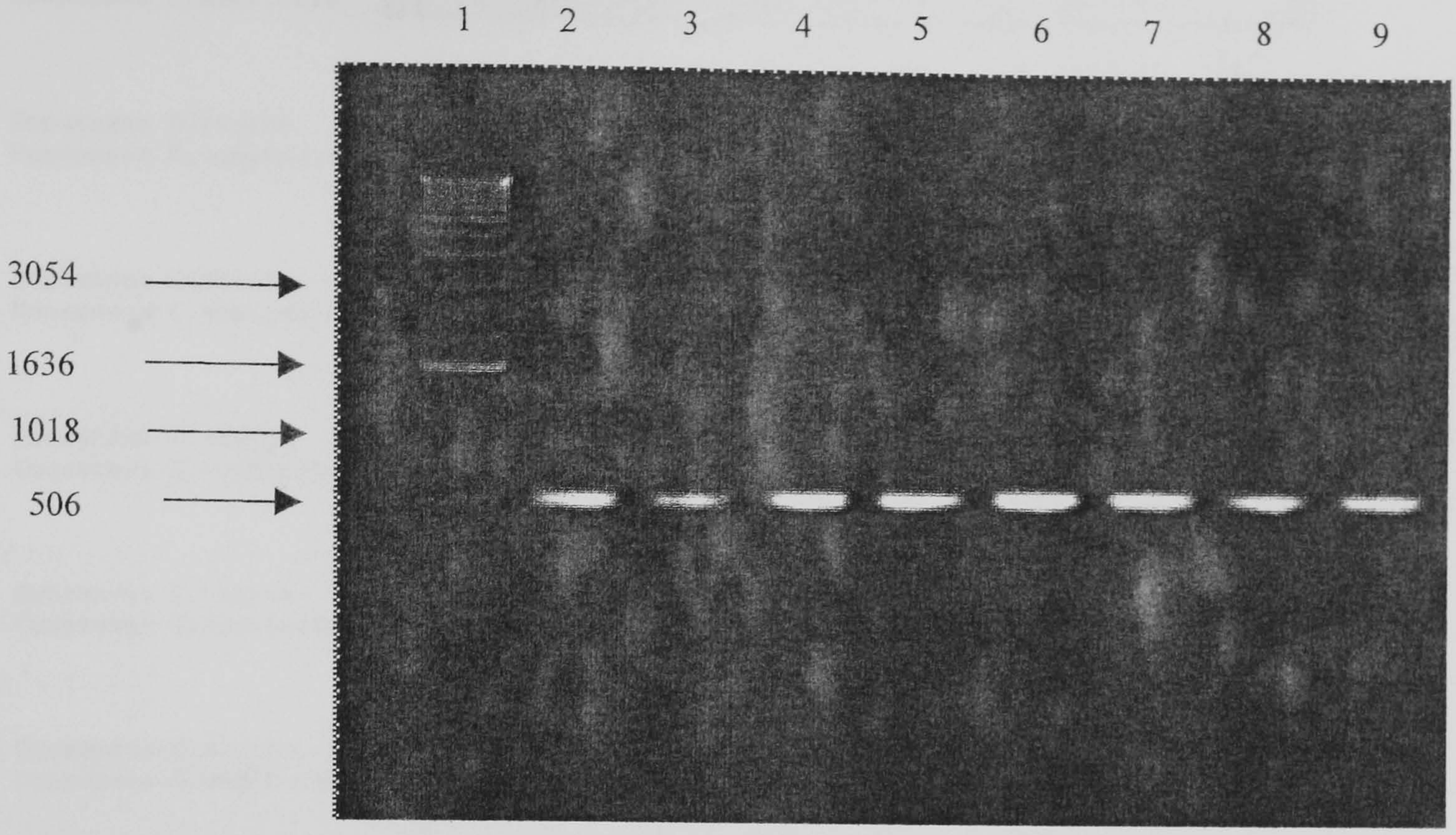


Figure 6.2. Agarose gel of Cytochrome oxidase I amplicons using primers C1-J-1718 and C1-N-2191. Lane 1 is a 1kb ladder. Lanes 2 and 3 *C. vicina* adult samples. Lanes 4 and 5 *C. vicina* larvae. Lanes 6 and 7 *C. vomitoria* adult samples. Lanes 8 and 9 *C.vomitoria* larval samples.

Figure 6.2 also indicates that no difference in amplicon size exists between adult and immature specimens.

The forward and reverse sequences for each sample were initially compared and all sample sequences were then aligned using CLUSTAL X (Appendix II for all aligned samples). Consensus sequences for both species were produced (Figure 6.3). These consensus sequences have been deposited in GenBank (Accession numbers AY536642 and AY536643 for *C. vicina* and *C. vomitoria* respectively). Due to primer flare at the beginning of the sample chromatograms, the first 50 bases (approximately) were often unclear and so these were disregarded in all sample sequences to produce a definitive sequence of 414bp for samples of both species.

The base numbering in the alignment of all sample sequences (Appendix II) is relative to the consensus sequence established and is not an indication of location within the mitochondrial genome. However, the consensus sequences in Figure 6.3 indicate the comparable base numbering compared to the *D. yakuba* mitochondrial sequence (Clary and Wolstenholme 1985).

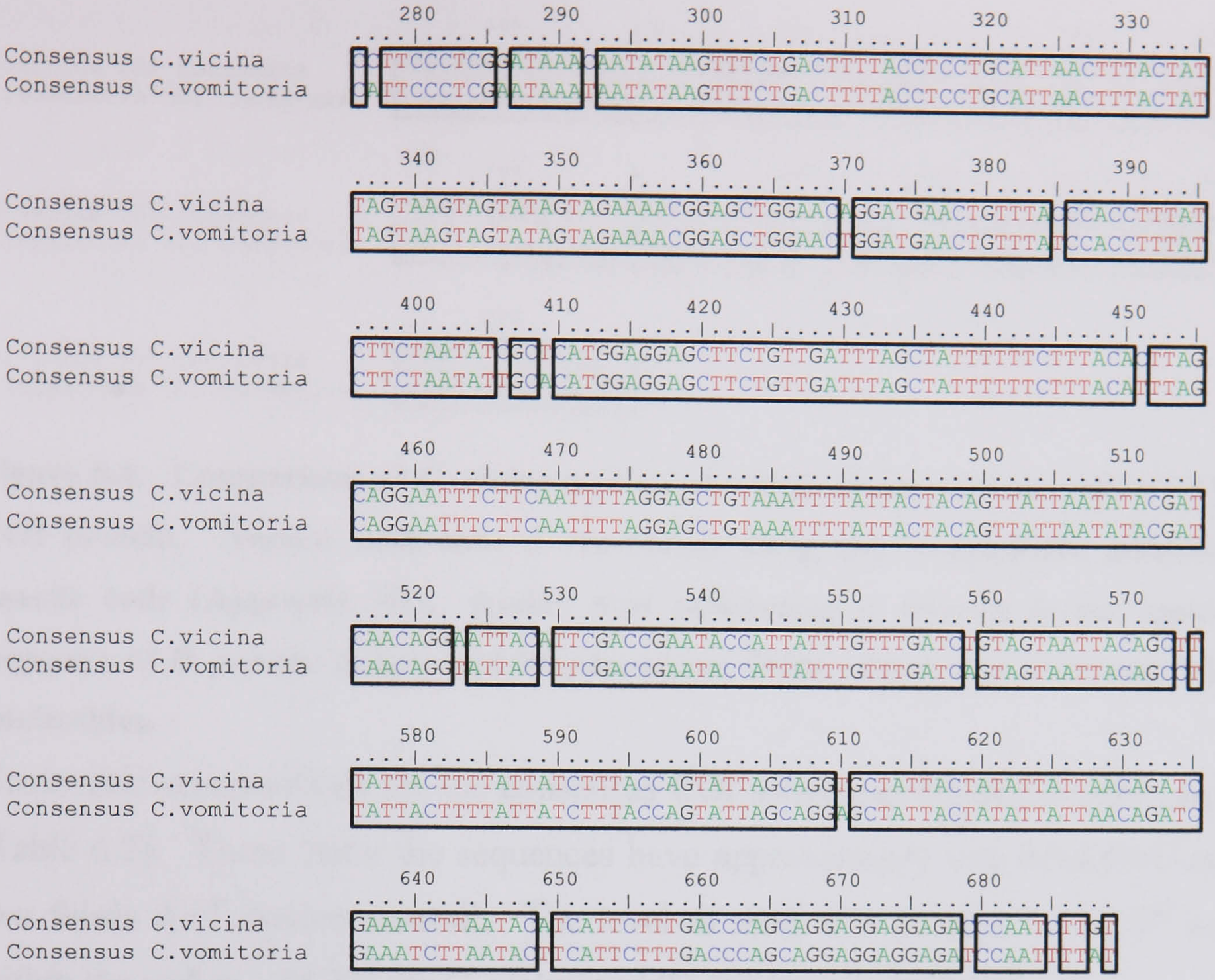


Figure 6.3. Aligned partial *C. vicina* and *C. vomitoria* mitochondrial cytochrome oxidase I sequences. The base numbering is relative to the equivalent *D. yakuba* COI sequence (Clary and Wolstenholme 1985). Black line surrounds identical nucleotides.

The consensus alignment indicates there are 17 interspecific differences for this region of the COI gene (Figure 6.3). These all consist of base substitutions (8 transversions and 9 transitions corresponding to 4.1% sequence divergence between species. Three substitutions are located in the membrane-spanning region compared to 6 in internal loops and 8 in external loop regions.

The nucleotide differences between species are all located in the 3rd position within the codon. As indicated in Figure 6.4 the nucleotide substitutions do not produce any differences in the amino acid sequences of *C. vicina* and *C. vomitoria*. The nucleotide sequence was translated using the invertebrate mitochondrial code as determined by Clary and Wolstenholme (1985). In common with other eukaryotic mitochondrial codes it shows some differences to the nuclear genomic code and is presented in full in Appendix III.

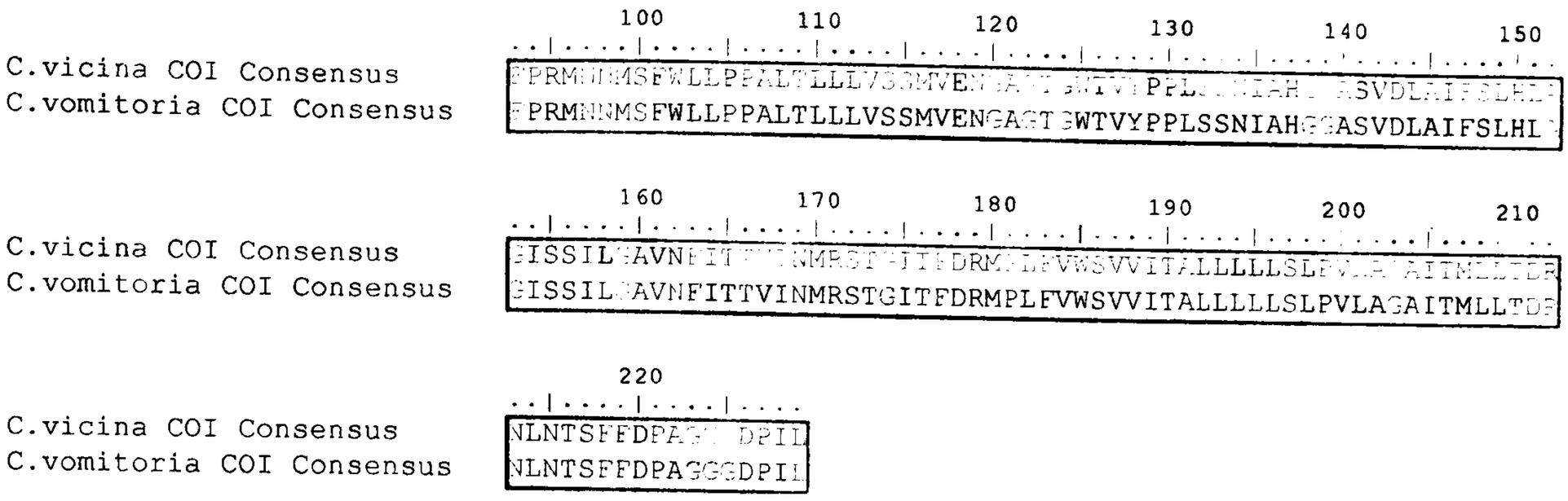


Figure 6.4. Comparison of *C. vicina* and *C. vomitoria* amino acid sequences of partial COI protein. Nucleic acid code is translated using the invertebrate mitochondrial genetic code (Appendix III). Amino acid numbering is relative to the amino acid sequence of *D. yakuba* (Clary and Wolstenholme 1985). Black line surrounds identical nucleotides.

Nucleotide compositions for the consensus COI sequences produced were calculated (Table 6.2). These show the sequences have approximately one third G+C content, two thirds A+T content overall. The level of A+T is very high in the 3rd position within the codon ~ 88.3% for *C. vicina* and 93.4% for *C. vomitoria*.

Table 6.2. Percentage values for the different nucleotides within the cytochrome oxidase I region for consensus sequences of *C. vicina* and *C. vomitoria*.

		A	C	G	T	%A	%C	%G	%T
C. vicina	1st Base in Codon	38	19	39	42	27.5	13.7	28.3	30.5
	2nd Base in Codon	17	42	22	57	12.3	30.4	16	41.3
	3rd Base in Codon	68	14	2	54	49.3	10	1.4	39
	Sequence Total	123	75	63	153	29.7	18.1	15.2	37
C.vomitoria	1st Base in Codon	38	19	39	42	27.5	13.7	28.3	30.5
	2nd Base in Codon	17	42	22	57	12.3	30.4	16	41.3
	3rd Base in Codon	70	9	0	59	50.7	6.5	0	42.7
	Sequence Total	125	70	61	158	30.2	17	14.7	38.1

6.2.2 Intraspecific variation

There are also intraspecific differences in the samples sequenced (Table 6.3). Of the 223 individuals in this study, only 20 show variation from the consensus sequence for the particular species (9%). The highest level of intraspecific variation within an individual sample is 2 out of 414 nucleotides (0.48%) and this occurs in *C. vicina* Cheltenham sample 2 and *C. vicina* Waterloo sample 5.

Table 6.3. Intraspecific variation in the partial cytochrome oxidase I gene. Sequence translated using invertebrate mitochondrial genetic code (Appendix III) [a] with reference to the overall structure of the COI protein. Ch = Cheltenham; Wa = Waterloo; Wi = Wimbledon; WiC = Wimbledon Common; HH = Hampstead Heath; BH = Box Hill; ES = East Sheen Common; PW = Petts Wood

Position within sequence	Structural Region[a]	Sample Name	Change from Consensus	Position of Nucleotide within Codon	Amino Acid Change
332	M3	*C. vicina* Ch 2	C>T	1st	L>F
332	M3	*C. vomitoria* 2	C>T	1st	L>F
332	M3	*C. vomitoria* 3	C>T	1st	L>F
332	M3	*C. vomitoria* 5	C>T	1st	L>F
332	M3	*C. vomitoria* 6	C>T	1st	L>F
332	M3	*C. vomitoria* 7	C>T	1st	L>F
332	M3	*C. vomitoria* L6	C>T	1st	L>F
332	M3	*C. vomitoria* 50	C>T	1st	L>F
332	M3	*C. vomitoria* WiC 3	C>T	1st	L>F
332	M3	*C. vomitoria* HH 3	C>T	1st	L>F
332	M3	*C. vomitoria* BH 2	C>T	1st	L>F
332	M3	*C. vomitoria* ES 4	C>T	1st	L>F
478	M4	*C. vicina* Wi 7	A>G	3rd	G>G
478	M4	*C. vicina* Wi 10	A>G	3rd	G>G
508	I2	*C. vomitoria* PW 3	T>C	1st	N>N
556	I2	*C. vicina* Wa 5	A>G	3rd	W>W
661	E3	*C. vicina* Wi 1	C>T	2nd	T>I
661	E3	*C. vicina* Wi 2	C>T	2nd	T>I
661	E3	*C. vicina* Ch 1	C>T	2nd	T>I
661	E3	*C. vicina* Ch 2	C>T	2nd	T>I
661	E3	*C. vicina* Wa 5	C>T	2nd	T>I
661	E3	*C. vomitoria* 15	C>T	2nd	T>I

As Table 6.3 demonstrates some of this intraspecific variation causes a change in amino acid sequence. This occurs when the nucleotide variation is either in the 1st or 2nd position in the codon.

6.2.3 Restriction enzyme analysis

Restriction maps were created using BioEdit software and are presented in Figure 6.5. For clarity in the maps presented, only enzymes that differ between the two species are shown. Restriction enzymes that cut at varying number of sites between the two species were assessed by examining where they cut and whether the theoretical fragment sizes produced would provide viable differentiation between species. Enzymes that cut in only one of the species were excluded, as those species that did

not contain the enzyme cut site would not be distinguished from those where enzyme digestion had not occurred.

Figure 6.5a. Restriction enzyme map for *C. vicina* cytochrome oxidase I partial gene region. Map shows enzymes that only cut the *C. vicina* DNA sequence in the positions shown and do not cut the *C. vomitoria* DNA sequence. Numbers indicate nucleotide cut sites relative to the total 523bp sequence.

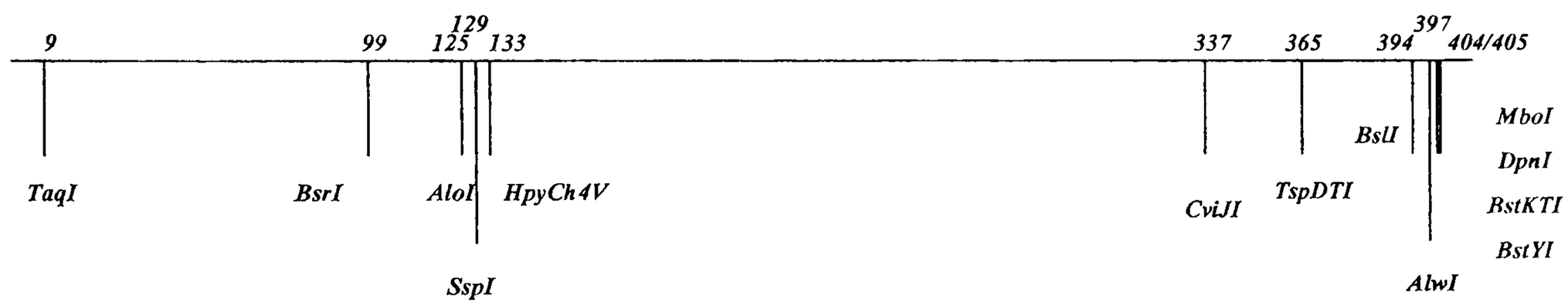


Figure 6.5b. Restriction enzyme map for *C. vomitoria* cytochrome oxidase I partial gene region. Map shows enzymes that only cut the *C. vomitoria* DNA sequence in the positions shown compared to the *C. vicina* DNA sequence. Numbers indicate nucleotide cut sites relative to the total 523bp sequence.

Most of the 22 potential enzymes were discounted after examination of the theoretical fragment patterns produced by each digestion. The only two possibilities were *Sfc*I or *Tsp*DTI and of these *Sfc*I was chosen to use in this work. The *Sfc*I cut site is 5' C↓TRYA↑G 3'. The theoretical fragment lengths after *Sfc*I digestion of the amplified gene regions are 63, 239 and 273 base pairs for *C. vicina*, 250 and 273 base pairs for *C. vomitoria*.

After digesting samples with *Sfc*I enzyme, the products were separated on a 1% agarose gel to visualise fragments (Figure 6.6). This contains an example of both species digested and undigested fragments.

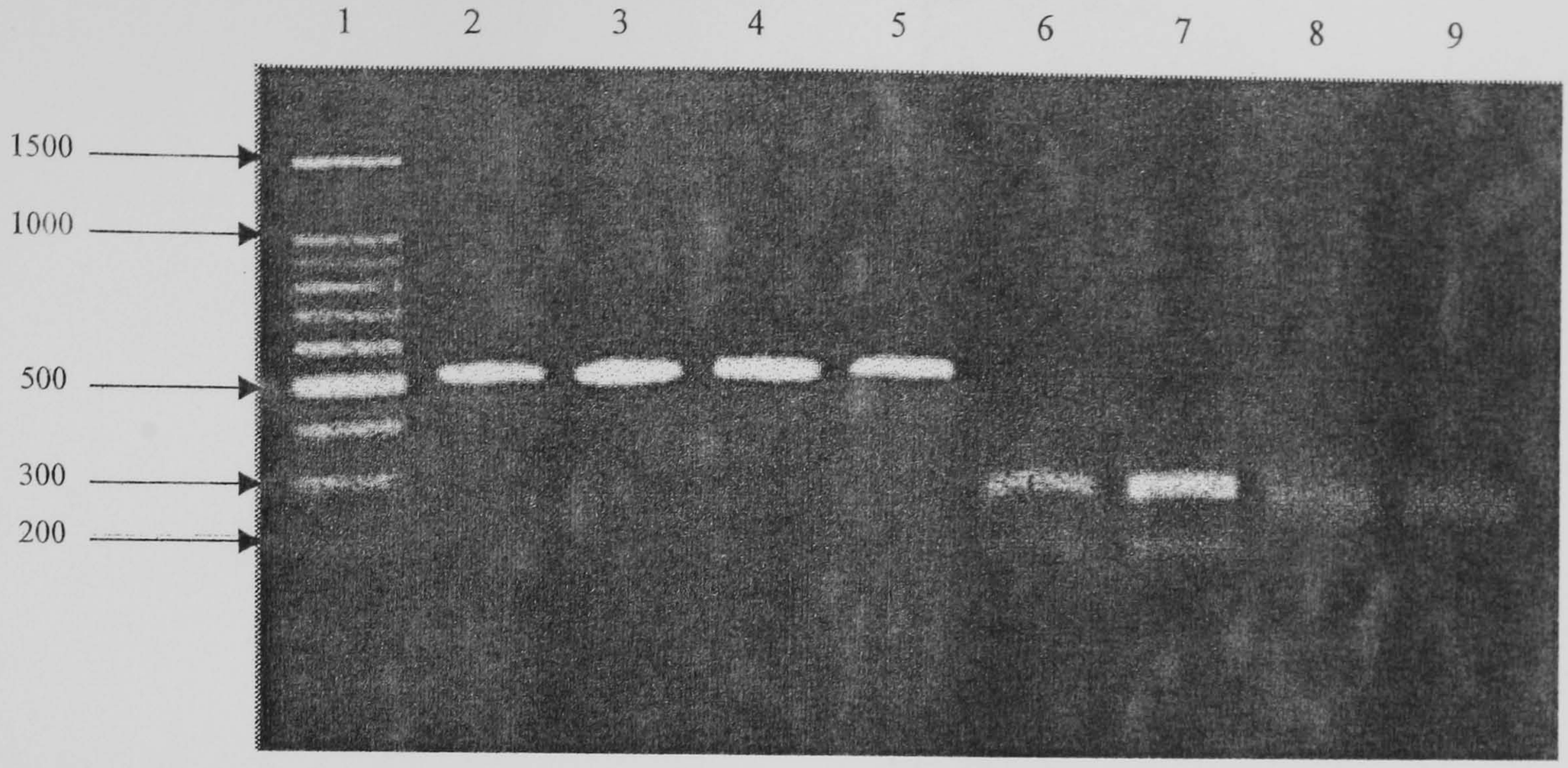


Figure 6.6. *Sfc*I restriction enzyme digest of COI amplicons. Lane 1 contains a 100bp ladder. Numbers represent number of DNA basepairs. Lanes 2 and 6 – undigested and digested *C. vicina* replicate 1 COI partial gene region. Lanes 3 and 7 - undigested and digested *C. vicina* replicate 2 COI partial gene region. Lanes 4 and 8 - undigested and digested *C. vomitoria* replicate 1 COI partial gene region. Lanes 5 and 9 - undigested and digested *C. vomitoria* replicate 2 COI partial gene region

The gel illustrates that the two species produce different fragment patterns after digestion and electrophoresis. It should also be noted that the two fragments in the *C.vomitoria* samples do not separate on the 1% gel and thus appear as one thick band.

6.2.4 Phylogenetic analysis

A neighbour-joining tree was constructed from the *C. vicina* and *C. vomitoria* sequences produced in this work (Figure 6.7). This indicates a clear separation of the two species. However, the populations do not show distinct separation within each species. Comparison with other Calliphoridae COI sequences deposited in GenBank is presented in Appendix IV. From this alignment a tree was produced (Figure 6.8). This tree separates the samples into clear genera with most species also showing distinct topologies.

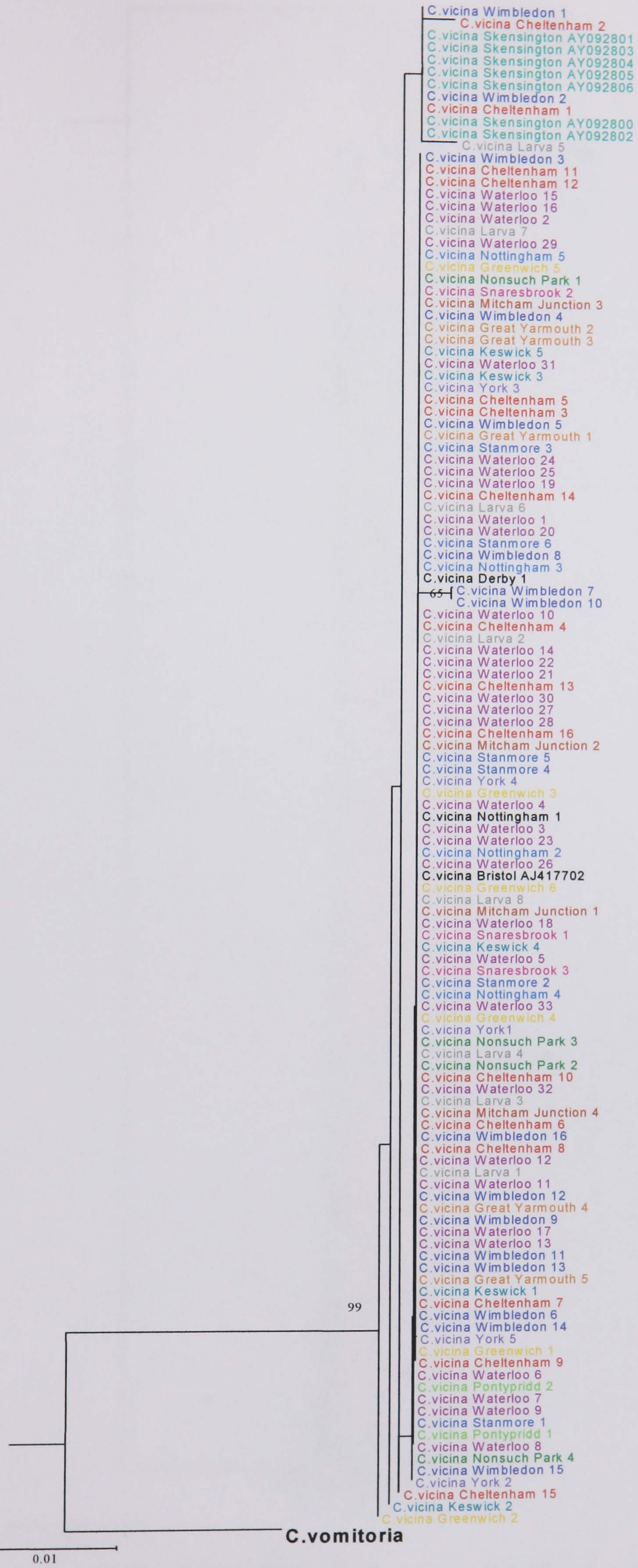


Figure 6.7. Neighbour joining phylogram of COI alignment. Figures represent bootstrap values after 1000 replicates. The tree is rooted on *L. sericata* sequence. *C.vomitoria* topology condensed to fit all *C. vicina* topology on one page.

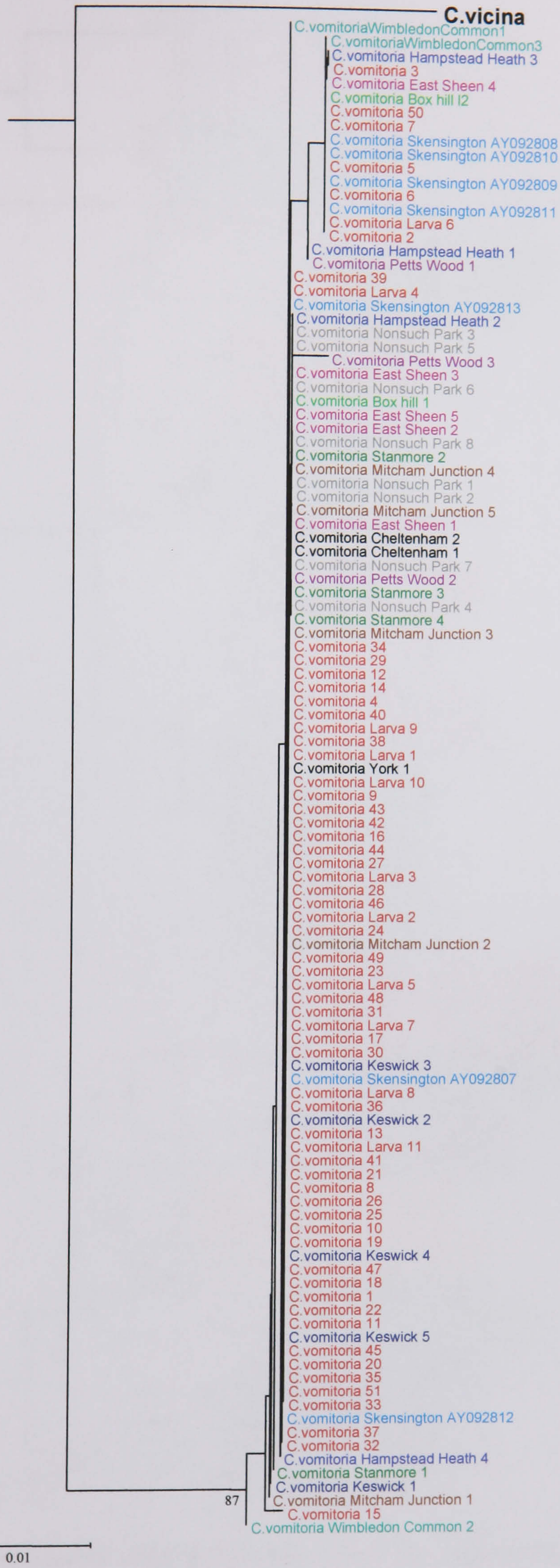


Figure 6.7 continued. Neighbour joining phylogram of COI alignment. Figures represent bootstrap values after 1000 replicates. The tree is rooted on *L. sericata* sequence. *C. vicina* topology condensed to fit all *C. vomitoria* topology on one page.

Figure 6.8. Neighbour joining phylogram based on COI alignment of Calliphoridae species deposited in GenBank. Accession numbers are shown in parentheses. Values in represent bootstrap values. The tree is rooted on a *D. yakuba* sequence.

6.3 Discussion

The cytochrome oxidase I primers C1-J-1718 and C1-N-2191 designed by Simon *et al.* (1994) are located in conserved insect mitochondrial areas and are named according to the 3' positions of the primers within the *D. yakuba* sequence. The theoretical size of the amplicon using these primers is 523 base pairs. This work shows that the amplicons for both *C. vicina* and *C. vomitoria* are of similar size. The DNA extraction technique will extract both nuclear and mitochondrial DNA. After sequencing it is apparent that due to differences in the genetic code between nuclear and insect mitochondrial DNA, these primers amplify part of the mitochondrial genome. In the nuclear genome code UGA codes for stop/termination codons which naturally are located at the ends of coding regions, whereas in the invertebrate mitochondrial code it codes for the amino acid tryptophan. The sequences produced in this study contain three codons for tryptophan. This agrees with Smith *et al.* (1996) who also deduced that their COII sequence was mitochondrial in origin as it contained five UGA codons. Alternatively a different extraction technique could be utilised that solely extracts mtDNA (Zhang and Hewitt 1996a).

The position of the amino acid sequence derived in this work within the cell membrane can be ascertained by comparison of the *D. yakuba* COI amino acid sequence as described by Clary and Wolstenholme (1985) along with the known structure of the protein (Figures 6.1 and 6.4). As anticipated, the amino acid sequence from this work starts within the first internal loop (I1), covers the structural regions M3, E2, M4, I2, M5 designated by Lunt *et al.* (1996) and finishes at the end of the external loop 3.

6.3.1 Interspecific variation

Evaluation of this partial COI region revealed 17 species specific base differences which is equivalent to 4.1% variation between *C. vicina* and *C. vomitoria*. This is in accordance with the work of Wells *et al.* (2001b) who found an interspecies difference of greater than 3% between the flesh flies *Sarcophaga argyrostoma* (Robineau-Desvoidy) and *Sarcophaga crassipalpis* Macquart. It is also of similar magnitude to Wells and Sperling's work on *Chrysomya* species (Wells and Sperling 1999, 2001). Schroeder *et al.* (2003) examined the COOH end of the COI region using *C. vicina* and *C. vomitoria* from Germany. They indicated a divergence of 4.3% between the

two species, similar to that of this study. The region chosen in the German study covered the structural regions I4, M9, E5, M10, I5, M11 and E6. As described at the beginning of this Chapter, the COI gene shows differing amounts of variability along its length. According to Zhang and Hewitt (1997a), there are two regions of high variability; the first is covered in this work and the German study covers the second. It can be seen therefore that these regions are of similar variability between these two species.

Harvey *et al.* (2003) only found three nucleotide differences between *C. dubia* and *C. augur* using a COI region (1.1%). The lack of variability between these two species could be due to the COI region that was examined or it could be that these two *Calliphora* species are more closely related than *C. vicina* and *C. vomitoria*. Malgorn and Coquoz (1999) found a level of 1.97% variation between *L. illustris* and *L. caesar*, which are also two closely related species. Wells *et al.* (2004) found that the COI gene could not be used to differentiate between *Chrysomya putoria* and *Chrysomya chloropyga* from areas where both flies cohabit.

These were no differences between all adults and all larvae for both species. This was as expected, the nature of DNA molecular markers means that they will be constant throughout all stages of the lifecycle. This allows an entomologist to differentiate between the *C. vicina* and *C. vomitoria* immature forms that are more difficult than adults to distinguish morphologically. This supports Sperling *et al.* (1994), who first demonstrated that mtDNA COI sequences (along with COII and $tRNA^{leu}$) could be used to identify the immature larvae of other forensically interesting Diptera (*L. illustris, P. regina* and *L. sericata*).

It is evident that this gene fragment is long enough to allow differentiation of two related species (414bp). It is important to balance the length of sequence required to report species differences and the length of fragment practically obtainable from forensic samples. Naturally, the ideal scenario would be to sequence the whole of the gene (or even the whole of the mtDNA genome) but often crime scene DNA is degraded and only short fragments can be amplified.

Harvey *et al.* (2003) note that the 278bp COI fragment sequenced in their study, whilst showing potential for identification (three base pair differences), would need to be extended to distinguish the closely related *C. augur* and *C. dubia*.

Another reason for utilising gene fragments that are not too small is to allow PCR-RFLP to be carried out. After restriction enzyme digestion, the resulting fragments will be difficult to visualise on a standard agarose gel if they are too short.

The differences between *C. vicina* and *C. vomitoria* partial COI DNA sequences in this study are all base substitutions - there are no insertions/deletions. This is in accordance with the work of other researchers on this region and is as expected in a coding sequence where an 'indel' would cause a frameshift most likely producing a lethal mutation.

All interspecific base substitutions are in the 3rd base within the codon. Due to the degeneracy of the genetic code the changes to the DNA sequence are all synonymous – the amino acid sequence is not altered. The high level of variability in the third codon position is typical of gene sequences under functional constraint as it is most likely to be synonymous.

The base substitutions are not equally distributed along the gene region length. A chi-square test was conducted which rejected the null hypothesis that the 17 differences were equally distributed throughout the seven structural regions (I1–E3) at a 5% significance level (χ^2=18; df=6; p<0.01). This disagrees with the findings of Stevens *et al.* (2002) for *Lucilia* species where nucleotide variation was evenly spread across the genes (COI and COII). Whilst this does however correspond with Lunt *et al.* (1996), the changes in DNA sequence found in this study did not produce any changes in the amino acid sequence whereas Lunt *et al.*'s conclusions on variability along gene length were based upon non-synonymous changes. It is worth noting that the exact positioning of variation in the current study does not concur with the findings of Lunt *et al.* (1996). They suggest that of the regions used in this study that M3, E2, and I2 would be the most variable and I1 is highly conserved. In this work M3 showed no variation and I1 had three base substitutions. This work illustrates that the membrane spanning regions showed the least variation, followed by the internal loops, with the external loops containing the majority of the variation. It should be stressed again that whilst it would be expected that the membrane spanning regions would be the most conserved as they would be under the greatest functional constraint, the current work can not be used to judge COI protein variation as there was no difference in amino acid sequence between the two species.

The *Calliphora* sequences produced in this study have an expect value in the order of 6 e^{-169} with the *Lucilia* sequences in GenBank. This indicates the high similarity of the Calliphoridae COI sequences. On comparison with other UK *Calliphora* COI sequences from GenBank the 17 interspecific differences discovered are present in the other *C. vicina* and *C. vomitoria* samples (Appendix IV). When comparing sequences using the NCBI BLAST facility all *Calliphora* sequences produce an expect value of 0.0. The *C. vicina* consensus established in this work has a 100% match with one sequence (AJ417702, Bristol UK) and 99.75% match with the other seven *C. vicina* sequences in the database. The one base pair difference between these and the consensus sequence is a C<>T transition at position 661 (numbering compared to *D. yakuba*). This is a nucleotide that showed intraspecific variation (C<>T) in the samples in this work (Table 6.3). This indicates that variation in this position exists in the general population.

The *C. vomitoria* consensus sequence for this study has a 100% homology with three sequences and 99.75% match with the four other *C. vomitoria* sequences in GenBank. The one base pair difference is the C<>T transition at nucleotide 332 referred to in Table 6.3 that was apparent in twelve specimens in this study.

For *C. vicina* the A+T nucleotide content of this region is 66.7% and for *C. vomitoria* it is 68.3%. The A+T content of the third codon position is much higher (88.3% for *C. vicina* and 93.4% for *C. vomitoria*). This is comparable to other research on this gene. Otranto *et al.* (2003) showed that for the COOH end of the COI gene typical A+ T base composition for Oestridae species were 67.9% for the total region and 82.5% for the third base codon position. Clary and Wolstenholme (1985), after sequencing the mitochondrial genome of *D. yakuba* in its entirety, found that for the protein coding genes, 93.8% of all codons ended with an A or T with a value of 91.6% for the COI gene. Crozier *et al.* (1989) examined the COI region of the honeybee (*Apis mellifera* Linnaeus) and found the total A+T content to be higher (76%) than both *Drosophila* (68%) and *Calliphora* species. This is also highlighted in the work of Lunt *et al.* (1996) who compared two species of Orthoptera, six dipteran species and one Hymenoptera (*A. mellifera*) and they noted that all had an A+T% of around 69% compared to 76% of *A. mellifera*.

6.3.2 Intraspecific variation

Some of the samples within this study showed a degree of intraspecific variation. The amount was very low. A total of 20 different individuals out of 223 showed nucleotide differences to the consensus sequences. The highest level within one individual was two bases out of 414 (0.48%). These intraspecific results are similar to those found by other researchers. Wells and Sperling (2001) reported an intraspecific level of variation within the COI and COII sequence for Chrysomyinae flies of below 1%. Likewise, Otranto and Stevens (2002) who used COI to distinguish between *Hypoderma* larvae reported a level of intraspecific variation of 0.30%. Zehner *et al.* (2004) also found a level of below 1% variation within flesh fly species, however they did only examine two specimens per species.

Whilst most researchers have also found that the intraspecific level of variation is too low on COI to resolve any relationships within the species level, Frati *et al.* (2000) found that for the collembolan *Orchesella chiantica* Frati and Szeptycki a population difference was evident. The gene region could distinguish a population of the Apennine Mountains from Tuscan populations.

Twelve samples had a C>T transition at nucleotide position 332. The samples were not all from the same location; indeed one sample is *C. vicina* and the rest *C.vomitoria*. Whilst seven out of twelve are *C. vomitoria* from a commercial supplier the inclusion of other *C. vomitoria* caught in the wild (Wimbledon Common, Hampstead Heath, Box Hill, East Sheen) indicates that it is not simply a case of the individuals being siblings, from the same maternal lineage. Interestingly the wild *C.vomitoria* showing this intraspecific variation are all from the greater London area. This variation was not seen in the *C. vomitoria* from elsewhere in England. However, this experiment indicates that whilst it appears in fairly low levels within the general London population (four samples out of 34 from the London area show this variation) more samples would need to be examined before conclusions could be drawn as to whether this variation was a SE England phenomena.

The change in DNA sequence is the first base position in the codon and is non-synonymous – the amino acid leucine in the established consensus sequences for both

species is changed to phenylalanine. Using the amino acid difference formula given by Grantham (1974) and the class boundaries and definitions of Li *et al.* (1985), leucine versus phenylalanine is a 'conservative' amino acid change (D=22). The amino acid D values compare polarity, composition and molecular volume between two amino acids to predict whether the change will affect the overall protein structure radically.

The T substitution at position 332 appears in other Calliphoridae species COI sequences deposited in GenBank (see Appendix IV for alignment). As mentioned previously, four other *C. vomitoria* sequences in GenBank from South Kensington, London also have a T at position 332. This agrees with the observation of it being variation linked to the SE of England.

All *C. vicina* have C at this position in the sequence. However, whilst *C. albiceps, C. marginalis* and *C. putoria* have a C, *C. megacephala* and *Chrysomya varipes* (Macquart 1851) have a T. Within the *Lucilia* genus, *L. ampullacea*, *L. cuprina* and *L. sericata* have a T, *L. illustris* has a C and *L. caesar* has both. Variation at this point is therefore widespread throughout Calliphoridae and even closely related species can differ. This variation presumably existed in the common ancestor of the Calliphoridae; otherwise the mutation would have had to occur independently in all the mentioned species after divergence. The leucine to phenylalanine amino acid substitution must be a neutral mutation as indicated by the D value of Grantham (1974).

Two *C. vicina* from the Wimbledon laboratory population have a synonymous nucleotide transition at base 478. There were 16 samples from the Wimbledon population included in this part of the study, so this intraspecific variation is therefore not indicative of a *C. vicina* Wimbledon population. Indeed as these two samples came from the same egg batch laid onto the trap's bait it is likely that they have come from the same mother and would therefore have the same maternal mtDNA. For a DNA marker to be indicative of a population, independent specimens (from different mothers) need to have the specific version of the marker indicating that this mutation happened many generations ago and has now spread through the population.

The other samples from Wimbledon included here came from different mothers and do not have the mutation at base 478. Whilst it is important to note this mutation

exists within the Wimbledon population it cannot be demonstrated by these results to be more than a difference on an individual level and is not representative of the total population.

Again this mutation exists within *Lucilia* and *Chrysomya* species. The adenine nucleotide is present in most species, but guanine appears as intraspecific variation in *L. caesar, L. illustris* and is indicative of *C. rufifacies* and *L. ampullacea* from the sequences deposited in GenBank.

Synonymous substitutions exist at nucleotide 508 in a *C. vomitoria* individual from Petts Wood and at base 556 in a *C. vicina* individual from the Waterloo laboratory population. Both of which are assumed to represent individual variation.

The last area of intraspecific variation in this gene region occurs at nucleotide 661 where six samples have a T nucleotide instead of the C present in the consensus sequence for both species. These include five *C. vicina* and one *C. vomitoria* samples. Similar to the '478' mutation, the *C. vicina* samples from Wimbledon are from the same egg batch. This mutation obviously exists in the general population of both species and will is not indicative of a particular population.

As mentioned previously, other *C. vicina* sequences deposited in GenBank also have a C>T substitution. When reviewing the GenBank *C. vicina* sequences, the majority of samples have a T residue. However, after communication with the researchers that deposited these samples it appears that the original samples are potentially from the same maternal lineage and therefore the number of GenBank sequences with the T variation at this nucleotide position is misleading in relation to the total UK population (S. Pickles, personal communication).

Thymine also appears as intraspecific variation in *L. sericata, L. caesar* and *L.cuprina* and is the common nucleotide in *L. illustris*, *C. megacephala,* and *C. marginalis* and is indicative of *C. albiceps.*

The mutation is unusual as it falls in the 2nd position within the codon. Any change in 2nd position of the insect mitochondrial codon will definitely result in a change of amino acid so naturally they are fairly rare. Frati *et al.* (2000) indicated that only 4.3% of variable sites in the COI region were located at the 2nd codon position compared with 79.7% in the 3rd codon position for *Orchesella* species. The amino acid is isoleucine instead of threonine in the *Calliphora* individuals. Using the amino

acid D values of Grantham (1974), this is classified as a moderately conservative amino acid change. It is likely that this change does not affect the overall protein significantly.

This does not concur with the work of Wells and Sperling (1999, 2001) who found that most cases of COI intraspecific variation were silent substitutions. They were however examining *C. rufifacies* and *C. albiceps*.

This region of intraspecific variation highlights a potential problem of using DNA molecular markers. This is the risk of not basing conclusions on a large enough sample size.

Considering the *C. vicina* samples in GenBank as an example - at position 661, seven out of eight (seven out of nine including this study) show T at this position compared to C for all the GenBank *C. vomitoria* entries. From this it could be assumed that this is another marker differentiating the two species. This will not be problematic if all further specimens are sequenced for identification (as the other 17 species specific markers could be used) but if a restriction enzyme was chosen that cut at this site *C.vicina* would be misidentified as *C. vomitoria*. After this study it can be concluded that a T at nucleotide at 661 is intraspecific variation to the normal C base as 115 out of 119 samples from various sources in this study have a C nucleotide in this position.

Stevens *et al.* (2002) used COI along with COII to show a high level of intraspecific variation between *L. cuprina* from Hawaii and those of other populations worldwide. Interestingly the authors noted the Hawaiian population shows greater similarity in this region with the consensus *L. sericata* sequence. This is highlighted in the Calliphoridae tree, where six *L. cuprina* are grouped with the *L. sericata* sequences. This could be a problem for identification techniques, which in this case would misidentify the Hawaiian population. Both these examples indicate the need to examine varying populations to check that molecular markers apply, especially if using markers predicted by other researchers as these may only truly apply to the region within which the researchers had taken their specimens. The need for varied geographical sampling was also highlighted by Harvey *et al.* (2003) who noted the COI divergence of *C. rufifacies* Australian populations.

This was taken into account when choosing a restriction enzyme for this study. Areas of known intraspecific variation were avoided because as mentioned, mutations at a

restriction site would cause misidentification if an enzyme cut/failed to cut unexpectedly. From the restriction enzyme possibilities, *Sfc*I was decided upon.

This restriction digest was shown to differentiate between *C. vicina* and *C. vomitoria* samples as illustrated in Figure 6.6. The pattern of fragments was not as expected theoretically with *C. vicina* showing two clear bands and *C. vomitoria* showing one band on an agarose gel. The 63bp product expected theoretically in the *C. vicina* samples is too small to be visualised as well as the other two bands. The two fragments in the *C. vomitoria* samples run together on the low percentage gel and so appear as one fragment. The one fragment for the *C. vomitoria* digestion products is not the same size as the undigested product. This therefore indicates that digestion has occurred and the sequences can be easily differentiated.

Schroeder *et al.* (2003) used PCR-RFLP on a 349bp COI region. Whilst they found that *C. vicina* and *C. vomitoria* could be distinguished using *Hinf*I enzyme, the *C.vomitoria* digestion fragment was uncut and the authors concluded that sequencing of this region was the best way to differentiate the species. They discovered that the *Hinf*I enzyme could differentiate *C. vicina*, *C. vomitoria* and *L. sericata* when digestion of the larger 1.3kb COI amplicon was used as the template for digestion. However, as discussed, a 1.3kb amplicon may not be experimentally viable for samples in varying degrees of DNA degradation.

The low level of intraspecific variation is highlighted by short branch lengths in the tree of *C. vicina* and *C. vomitoria* samples. This COI region does not provide sufficient variation to be able to distinguish the different populations of these species. However, as the Calliphoridae tree indicates, there is enough interspecific variation to not only distinguish between *C. vicina* and *C. vomitoria* but also some of the other Calliphoridae species. When examining the *Lucilia* species in this tree it can be seen, that unlike all other species which are grouped together on the respective branches, there is a *L. caesar* sample amongst the *L. illustris* samples and *vice versa.* Likewise six *L. cuprina* samples are located amongst the *L. sericata* sequences. The *L.caesar/L.illustris* grouping may simply be a case of misidentification as these sister taxa can be difficult to distinguish morphologically. The *L. cuprina* samples grouped within the *L. sericata* sequences have been discussed previously as a potential hybrid group (Stevens *et al.* 2002).

The tree also indicated the close relationship of *Lucilia* and *Calliphora* species – which lie together separate from the *Chrysomya* species.

This phylogenetic analysis was not performed to examine the evolutionary relationships between *C. vicina*, *C. vomitoria* and other Calliphoridae but as noted in Wells *et al.* (2001b), if there was not an exact match between an unknown crime scene insect exhibit and the consensus sequences for possible species and if the level and positions of intraspecific variation had not previously been assessed it would be advisable to include the unknown in a phylogenetic analysis to locate the closest relative. Whilst an exact match would not have been revealed, an investigator could use development timings for the closest relative to establish an approximate PMI.

In summary, the COI region examined in this study provides a means of distinguishing *C. vicina* from *C. vomitoria* samples. Whilst a level of intraspecific variation does exist within this region, samples from varying geographic locations indicate that the molecular markers established to separate *C. vicina* and *C. vomitoria* will work for unknowns collected within England and Wales. The sequence analysed could not distinguish between UK populations of *C. vicina* or *C. vomitoria*. Further work needs to be conducted to examine the COI sequence for other UK *Calliphora* species so that an entomologist could utilise this region to identify unknown *Calliphora* specimens.

The use of *Sfc*I in a restriction digest of the PCR amplicons offers a quick and inexpensive method of differentiating between these two species thus allowing high throughput for a forensic entomologist. The intraspecific variation observed after analysis of the Calliphoridae with the GenBank database indicated that more than one restriction enzyme would be required to differentiate between all Calliphoridae species.

Whilst it is accepted that complete alive or preserved samples can be distinguished morphologically, specimens are not always presented to the entomologist as such and thus this COI region would provide a means of dealing with crime scene unidentified insect evidence.

Chapter 7

Potential Molecular Marker – Xanthine dehydrogenase

Xanthine dehydrogenase (XDH) catalyses the hydroxylation of hypoxanthine and xanthine to form uric acid. This compound acts as an antioxidant in humans (Ames *et al.* 1981). *Drosophila* mutants (known as *rosy*), which cannot produce XDH, have been shown to be sensitive to active oxygen and consequently have shorter lifespans. In the larval forms of *Bombyx mori* Linnaeus the uric acid formed causes the epidermis to become opaque and often absence of this enzyme is lethal (Komoto *et al.* 1999 and references therein).

Whilst examining the *C. vicina* XDH gene for homology to the *D. melanogaster* gene, Rocher-Chambonnet *et al.* (1987) discovered that polymorphism existed within their *C. vicina* laboratory population. The polymorphism became apparent after digestion of the gene with the *Eco*RI restriction enzyme. Individual *C. vicina* flies either had one of two DNA bands or both when digestion products were separated using agarose gel electrophoresis. This indicated the presence of different XDH alleles within their *C. vicina* population. The variation between alleles must due to one or more nucleotide differences in the restriction enzyme cut site. The *C. vicina* XDH gene was later sequenced and the 3D protein structure deduced by Houde *et al.* (1989). The protein is translated from four coding regions.

This variation concurs with the earlier findings of Buchanan and Johnson (1983) who noted XDH polymorphism in *Drosophila* species. They observed the presence of different allozymes of XDH when separated on a gel. A level of 1.68% intraspecific variation has also been noted in the XDH gene of *Ceratitis capitata* (Wiedemann) (Pitts and Zweibel 2001).

The prevalence of the XDH polymorphism within populations of *C. vicina* and *C.vomitoria* was examined and assessed as to whether it could be used as an indicator of geographical population location. Chapter 6 examined a mitochondrial protein-coding gene and now a nuclear coding region would be focussed on. This region has

not been previously used as a potential marker for *Calliphora* variation, in a forensic context or otherwise.

7.1 Materials and Methods

7.1.1 Sample Preparation and DNA Extraction

Calliphora vicina and *C. vomitoria* adults from various locations were prepared and the DNA extracted using the techniques described in Section 5.2.

7.1.2 Primer Design

The *C. vicina* XDH DNA sequence produced by Rocher-Chambonnet *et al.* (1987) has been deposited in GenBank (accession number M18423). This sequence is only 480bp in length and after examining the sequence for *Eco*RI cut sites using BioEdit software, there were none. The authors had only sequenced a relatively small area of the gene region they had used in the restriction enzyme digests. This sequence was aligned with the XDH exon 2 sequence deposited by Houde *et al.* (1989), accession number X07323, and found to have 100% similar nucleotides. When the X07323 XDH nucleotide sequence was examined for *Eco*RI cut sites, there were found to be two, one at nucleotide 445 and one at 3164 (relative to the complete X07323 sequence). Another *C. vicina* XDH GenBank sequence (accession number X17106) was also aligned and after examination for *Eco*RI cut sites, a further cut site was evident at position 844 (relative to the X07323 sequence). This could be the restriction enzyme site which causes the polymorphism within the *C. vicina* XDH gene, commented on by Rocher-Chambonnet *et al.* (1987) as described previously.

Primers were designed using Primer3 online software as described in Section 5.3. The *C. vicina* complete sequence deposited in GenBank, accession number X07323, was used as a template for primer design. The primers were designed to flank the *Eco*RI cut sites (at nucleotides 445 and 3164) and would theoretically produce amplicons of 3210 bp. Table 7.1 indicates the primer sequences, XDH External Forward and XDH External Reverse I, used initially. However, the length of the amplicon (3.2kb) meant that amplifications were not always successful. Any degradation of DNA will limit the total length of DNA that can be amplified. Amplification of a shorter length of the XDH gene was required to ensure consistent results with the known samples in this work and also to make the method applicable for use on unknown samples collected

by forensic entomologists. Another reverse primer was designed (XDH External Reverse II in Table 7.1). This would produce a theoretical amplicon of 905bp and include the *Eco*RI cut site at nucleotide 445 and the alleged polymorphic cut site at position 844 (Figure 7.1). If the enzyme cuts at 445 only then the digestion fragments 87 and 818bp will be present. If it cuts twice then fragments of 87, 399 and 419bp will be present.

Table 7.1. Primers to amplify a partial region of the *C. vicina* and *C. vomitoria* xanthine dehydrogenase gene.

Primer Name	Sequence of Primer
XDH External Forward	5' CCCTCGATGAAAGATTTGGA 3'
XDH External Reverse I	5' AATACAATGGCCGGATTCA 3'
XDH External Reverse II	5' ATGGACCTGGGATCAATGAA 3'

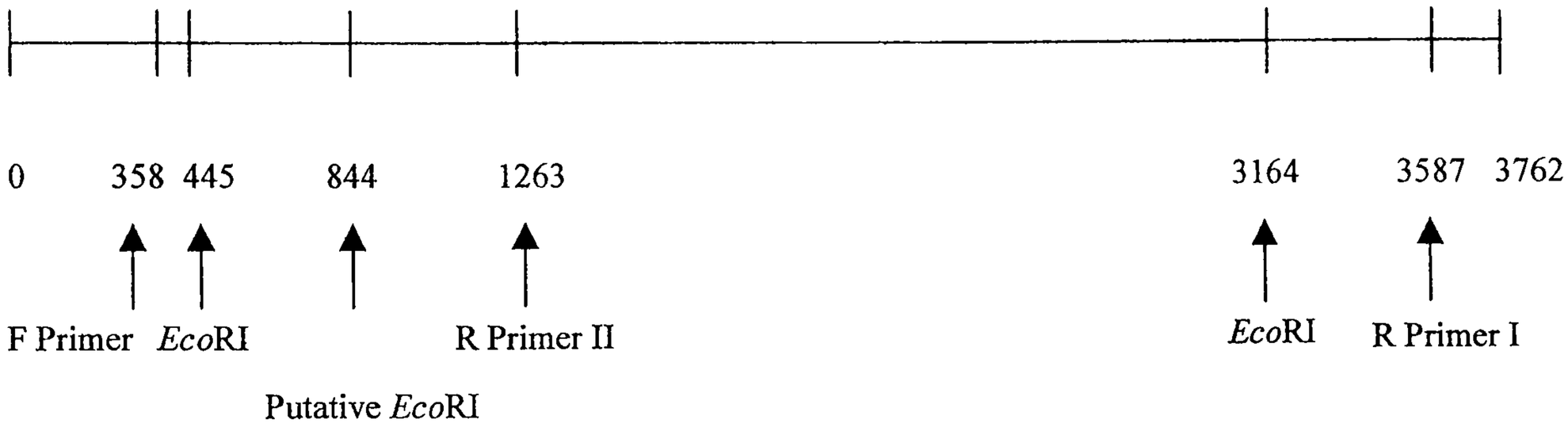


Figure 7.1. Diagram to illustrate the primer binding sites and the restriction enzyme cut locations relative to the 3762bp XDH *C. vicina* sequence GenBank Accession number X07323.

7.1.3 Amplification of a partial region of the XDH gene

Amplification was conducted as in Section 5.4. Samples were run on the following thermal cycle; 94°C for 2 min; followed by 32 cycles of [94°C for 30s; 55°C for 30s; 72°C for 1 min]; followed by 72°C for 7 min.

7.1.4 Amplicon Purification

After visualisation on à 1% agarose gel to check for amplification success, PCR amplicons were cleaned using GFX PCR Gel and PCR Purification Kit (Amersham

Biosciences) as described in Section 5.5. Samples were eluted off the spin column using 50µl ddH_2O.

7.1.5 Restriction Enzyme Analysis

Amplicons were digested with *Eco*RI (Promega, USA) according to the manufacturer's instructions. *Eco*RI cuts at the site 5' G ↓ AATT ↑ C 3'. The cut sites are located at 87 and 486 within the 905bp amplicon. Two microlitres of 10X Buffer H (supplied with enzyme, Promega, USA) was added to a sterile 1.5ml tube along with 0.2µl BSA (10µg/µl, supplied with kit). Approximately 1µg of DNA was added per reaction and samples made up to a total volume of 19.5µl with ddH_2O. PCR products were quantified approximately using a DNA ladder run alongside the samples on the gel. After mixing with a pipette, 0.5µl *Eco*RI (10U/µl) was added and the tubes were incubated at 37°C for three hours. Lambda DNA was used as a positive control as it has a known number of *Eco*RI cut sites (5). Negative 'no-enzyme' controls were also included along with the experimental samples.

Samples were run on an agarose gel, later stained with ethidium bromide to visualise the DNA fragments under UV as described in Section 5.2.4.

7.1.6 Sequencing

For samples that were to be sequenced, an internal region flanking the putative polymorphic site was amplified. This was to reduce the size of the amplicon to be sequenced (from 905 to 409bp). Internal primers were designed using Primer3 software (Table 7.2).

Table 7.2. Primers for amplification of an internal region of the partial XDH gene region

Primer Name	Sequence of Primer
XDH Internal Forward	5' TAAGGGCGAACGTGCTACTT 3'
XDH Internal Reverse	5' TCAACACGGATTCATATCG 3'

The external XDH amplicons were used as template DNA in these PCR reactions. The samples were run on the following thermal cycle; 94°C for 2 min; followed by 32 cycles of [94°C for 30s; 55°C for 30s; 72°C for 1 min]; followed by 72°C for 7 min.

Once the samples had been cleaned as in Section 5.5. They were either set up for sequencing on the 310 Genetic Analyser (Section 5.6) or were sequenced by Dr Peter Kabat and Dr Joanne Martin using a 3700 Genetic Analyser (Applied Biosystems, USA).

7.1.7 Data Analysis

Sequences obtained were checked by eye and then aligned using BioEdit software.
Data analysis of the XDH sequences produced in this work focussed upon both population structure and prediction of population variation.

Initially, an exact test of whether the populations were in Hardy Weinberg equilibrium was conducted. Traditionally χ^2 tests are conducted upon simple two allele systems, comparing observed and expected genotype frequencies. According to Guo and Thompson (1992) loci with multiple alleles require the use of exact tests. The Hardy Weinberg exact test was conducted by GENEPOP v.3.1 online software (wbiomed.curtin.edu.au/genepop/index.html) of Raymond and Rousset (1995) using the Markov chain method described by Guo and Thompson (1992) with the default parameters.

To assess whether any differences existed between the populations an AMOVA was also conducted. Populations containing only one sample had to be removed before calculation of AMOVA as there is no within population variance value for these populations. This calculation provides a F_{ST} statistic for codominant data and Φpt for haploid data analogous to Wright's F-statistics. The F-statistics in population genetics are a measure of the fixation index of alleles at a locus and are widely used to characterise population genetic structure. There are three F-parameters, F_{ST}, F_{IS} and F_{IT}.

F_{IS} is a measure of the inbreeding within a subpopulation and is analogous to the 'f' inbreeding coefficient of Weir and Cockerham (1984).

$$F_{IS} = (H_{EXP}-H_{OBS}) / H_{EXP}$$

Where H_{EXP} is total expected heterozygosity (calculation based upon random mating) and H_{OBS} is total observed heterozygosity.

F_{IT} is a measure of how the population structure has affected the overall heterozygosity of individuals within the total population and is analogous to the 'F' parameter of Weir and Cockerham (1984).

$$F_{IT} = (H_{TOTAL\ EXP}-H_{OBS}) / H_{TOTAL\ EXP}$$

F_{ST} indicates how differentiated the separated populations are and is analogous to the coancestry coefficient (θ) of Weir and Cockerham (1984).

$$F_{ST} = (H_{TOTAL\ EXP}-H_{EXP}) / H_{TOTAL\ EXP}$$

F_{ST} is zero when populations have identical allele frequencies and is equal to one when they are fixed for different alleles.

These F statistics can be used to calculate N_m (the number of migrants between populations per generation) by following formula.

$$N_m = [(1/F_{ST})-1]/4$$

Nucleotide distance measurements between the populations within both species were calculated with the Tamura-Nei correction factor method using MEGA v.3 software package as described in Section 5.7. These genetic distances between populations can be compared with geographic distances graphically in Microsoft® Excel and also statistically using the Mantel test for matrix correspondence in GenAlEx. This test provides a correlation coefficient (R_{xy}) between the two types of distance between populations and this coefficient is tested for its deviation from zero. This test uses Monte-Carlo strategy to overcome the problem of individual values in a distance matrix not being independent of one another. Each coefficient is tested against 999

random permutations. This is a test of the isolation by distance theory indicative of the stepping stone model; non-neighbours will show greater genetic differences.

Neighbour-joining trees were constructed from the pairwise distances established using MEGA software as described in Section 5.7.

The probabilities of samples being assigned to the correct populations were assessed. This was done using the 'Population Assignment' feature of the GenAlEx software package. This assessment is based upon the test of Paetkau *et al.* (1995, 2004) and produces log likelihood values of each sample for each population based upon allele frequencies. From these the most likely population can be assigned to each sample.

7.2 Results

7.2.1 *Eco*RI restriction enzyme polymorphism

Figure 7.2 illustrates examples of both the undigested fragment after amplification using the external XDH primers and the three types of product after digestion using the *Eco*RI enzyme.

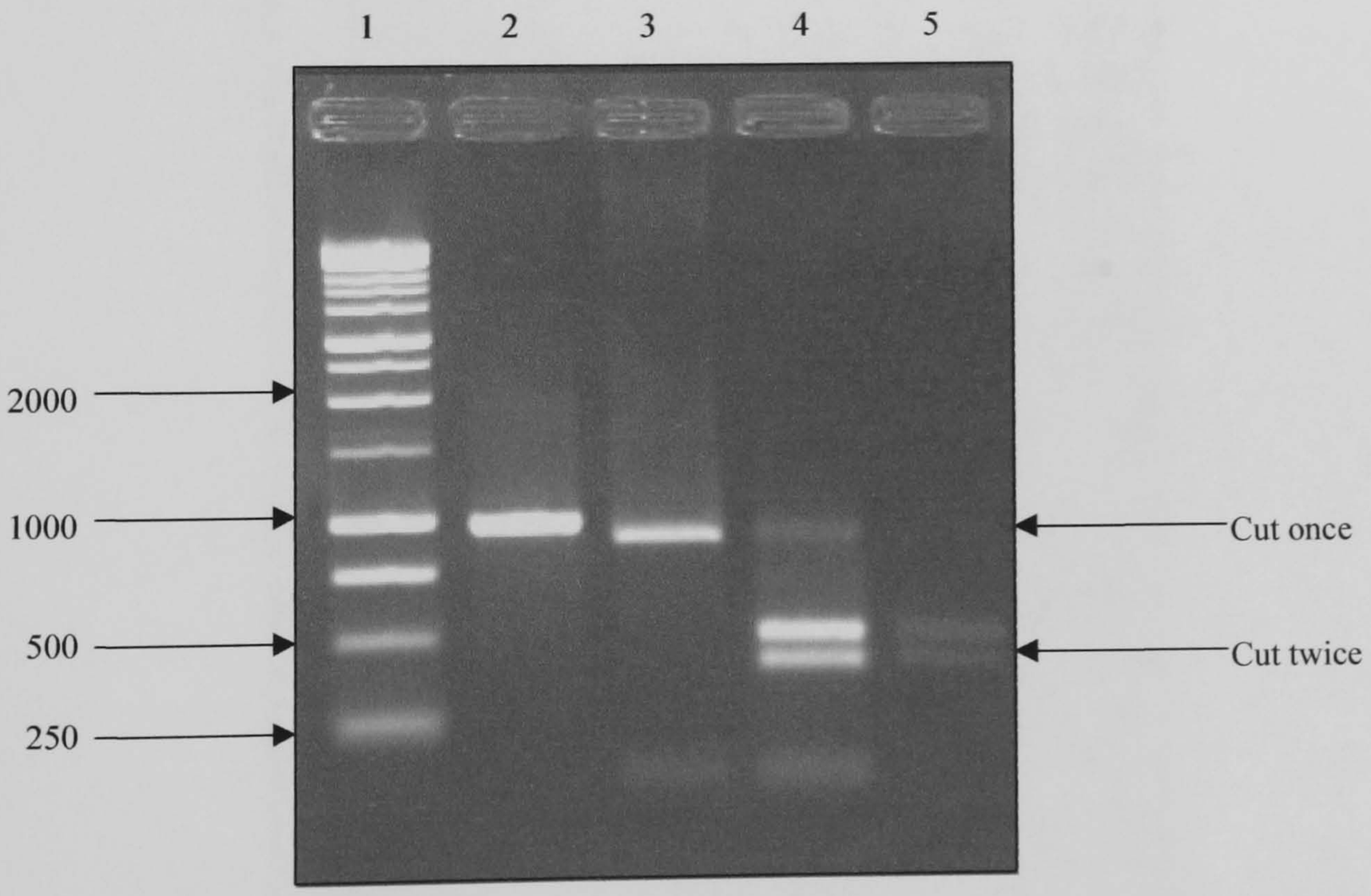


Figure 7.2. The XDH amplicon after *Eco*RI restriction enzyme digestion to illustrate the different alleles that are evident. Lane 1 contains DNA size ladder. Lane 2 contains the undigested XDH amplicon (905bp). Lane 3 contains the 87 and 818bp fragments. Lane 4 contains the 87, 399, 419 and 818bp fragments and Lane 5 the 87, 399 and 419 fragments.

The undigested fragment is 905bp. After digestion with *Eco*RI, three fragment patterns are evident within the *C. vicina* and *C. vomitoria* samples. The first is an 818bp fragment, slightly smaller than the undigested fragment (Lane 3, Figure 7.2). For this work this polymorphism has been nominated Genotype 'A'. The second (Lane 4) also contains this 818bp fragment along with two smaller fragments (399 and 419bp). This has been called Genotype 'AB'. The third (Lane 5) has just the two smaller fragments and is named 'B'. All genotypes also contain an 87bp fragment that is too small to be visualised well on this agarose gel. This fragment does not distinguish between genotypes, as it is present in all three. Genotypic frequencies of all samples used in this part of research are found in Table 7.3.

Table 7.3. Genotype frequencies relative to population location for both *C. vicina* and *C.vomitoria* based upon fragment patterns after digestion of XDH exon 2 amplicons using *Eco*RI restriction enzyme. Genotype A = 818bp fragment Genotype B = 399 and 419bp fragments and Genotype AB 399, 419 and 818bp fragments.

			Genotype		
	Population	n	A	B	AB
C. vicina	Cheltenham	11	0.727	0.091	0.182
	York	5	1.000	0.000	0.000
	Nottingham	5	0.800	0.000	0.200
	Keswick	5	0.600	0.200	0.200
	Derby	1	1.000	0.000	0.000
	Gt Yarmouth	3	1.000	0.000	0.000
	Pontypridd	2	1.000	0.000	0.000
	Snaresbrook	5	1.000	0.000	0.000
	Mitcham Jn	5	1.000	0.000	0.000
	Nonsuch Pk	5	1.000	0.000	0.000
	Wimbledon	26	1.000	0.000	0.000
	Waterloo	8	0.875	0.000	0.125
	Stanmore	5	1.000	0.000	0.000
	Greenwich	5	1.000	0.000	0.000
C. vomitoria	York	1	0.000	1.000	0.000
	Keswick	4	0.000	1.000	0.000
	Pett Wood	5	0.000	1.000	0.000
	Nonsuch Pk	5	0.000	1.000	0.000
	Boxhill	5	0.000	1.000	0.000
	East Sheen	5	0.000	1.000	0.000
	Mitcham Jn	5	0.000	1.000	0.000
	Hampstead Heath	5	0.000	1.000	0.000
	Wimbledon Cn	5	0.000	1.000	0.000
	Stanmore	5	0.000	1.000	0.000
	Cheltenham	2	0.000	1.000	0.000
	Commercial	14	0.071	0.786	0.143

The results indicate that there is an obvious interspecies division present. The majority of *C. vicina* are Genotype A whereas the majority of *C. vomitoria* are Genotype B.

From Table 7.3 it is also clear that a level of polymorphism does exist within both the *C. vicina* and the *C. vomitoria* populations albeit at relatively low levels compared to the total number of samples of each species analysed (*C. vicina*, 7 out of 91 samples ~ 8% and *C. vomitoria*, 3 out of 61 samples ~ 5%). The polymorphism is however limited to a few of the populations. In the *C. vicina* Cheltenham population 3 out of 11 samples show the polymorphism, in the Nottingham population 1 out of 5 samples, in the Keswick population 2 out of 5 samples and in the Waterloo population 1 out of 8 samples has a different genotype to the majority. Only the laboratory population of *C. vomitoria* established from commercially obtained maggots showed the polymorphism in the samples tested in this study (3 out of 14 samples).

7.2.2 *C. vicina* and *C. vomitoria* XDH sequence comparison

Samples were then sequenced to examine the *Eco*RI polymorphism further and also to assess other variation. A smaller internal sequence was examined. The multiple alignments are presented in Appendix V. The nucleotide numbering in this alignment is relevant only to the fragment amplified using the internal primers designed in this work.

Consensus sequences for both species were established. These are presented in Figure 7.3 along with the *C. vicina* XDH sequence (X70323) upon which the primers for this study were designed. The base numbering of the consensus sequences is compared to the whole XDH exon 2 (Houde *et al.* 1989). These consensus sequences have been deposited in GenBank (accession numbers AY944493 and AY944494 for *C. vicina* and *C. vomitoria* respectively). From these consensus sequences the proportion of each nucleotide was established (Table 7.4). The ratio of A+T of the total sequence is nearly two thirds for both species (62.2 % for *C. vicina* and 61.7% for *C. vomitoria*).

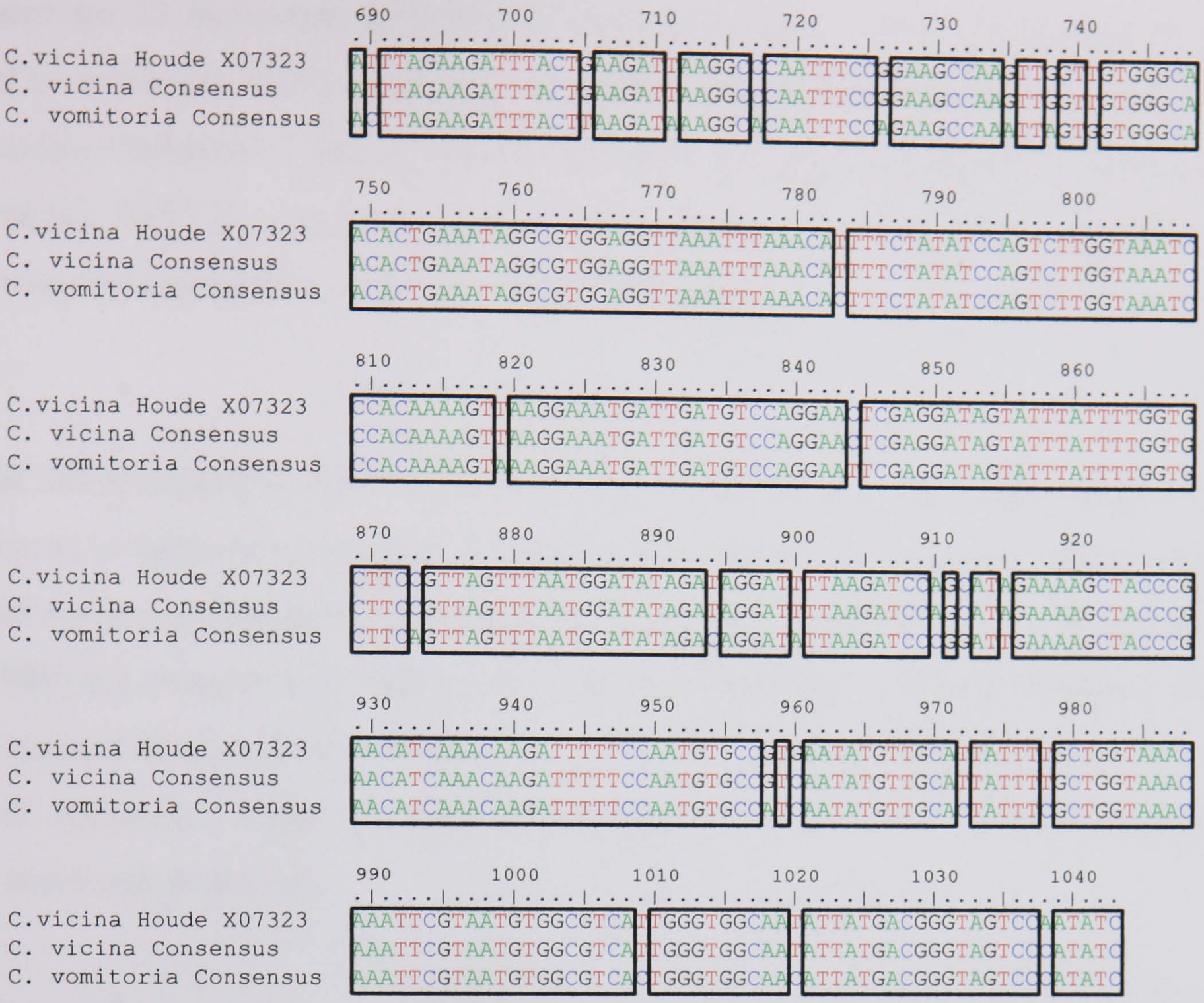


Figure 7.3. Alignment of partial XDH consensus sequences of *C. vicina* and *C. vomitoria*. Base numbering is relative to the entire *C. vicina* XDH Exon 2 as sequenced by Houde *et al.* (1989). Black outline surrounds identical nucleotides in all sequences.

Table 7.4. Nucleotide composition of *C. vicina* and *C. vomitoria* consensus partial xanthine dehydrogenase gene region.

		A	C	G	T	%A	%C	%G	%T
C. vicina	**1st Base in Codon**	39	18	38	23	33.05	15.25	32.2	19.5
	2nd Base in Codon	40	19	17	43	33.6	16	14.3	36.1
	3rd Base in Codon	32	18	24	44	27.1	15.3	20.3	37.3
	Sequence Total	111	55	79	110	31.3	15.5	22.3	30.9
C. vomitoria	**1st Base in Codon**	39	19	37	23	33.05	16.1	31.35	19.5
	2nd Base in Codon	40	19	17	43	33.6	16	14.3	36.1
	3rd Base in Codon	38	22	22	36	32.2	18.65	18.65	30.5
	Sequence Total	117	60	76	102	33	16.9	21.4	28.7

There are 22 nucleotide differences between the consensus sequences for *C. vicina* and *C. vomitoria* (6.2% variation). All differences are base substitutions; there are no insertions/deletions. The *C. vicina* consensus sequence has a 99.7% identity to the *C.vicina* X07323 sequence, with a nucleotide difference at position 1038. The *C.vomitoria* consensus sequence has a 93.8% match.

The DNA sequences were translated using the standard genetic code, as this gene is located in the nuclear genome. The alignment of the *C. vicina* and *C. vomitoria* amino acid consensus sequences along with the amino acid sequence derived by Houde *et al.* (1989) are presented in Figure 7.4. The one nucleotide difference between the two *C.vicina* DNA sequences (Figure 7.3) does not cause any differences in the amino acid sequence. There are three amino acid differences between the *C. vicina* and *C.vomitoria* sequences.

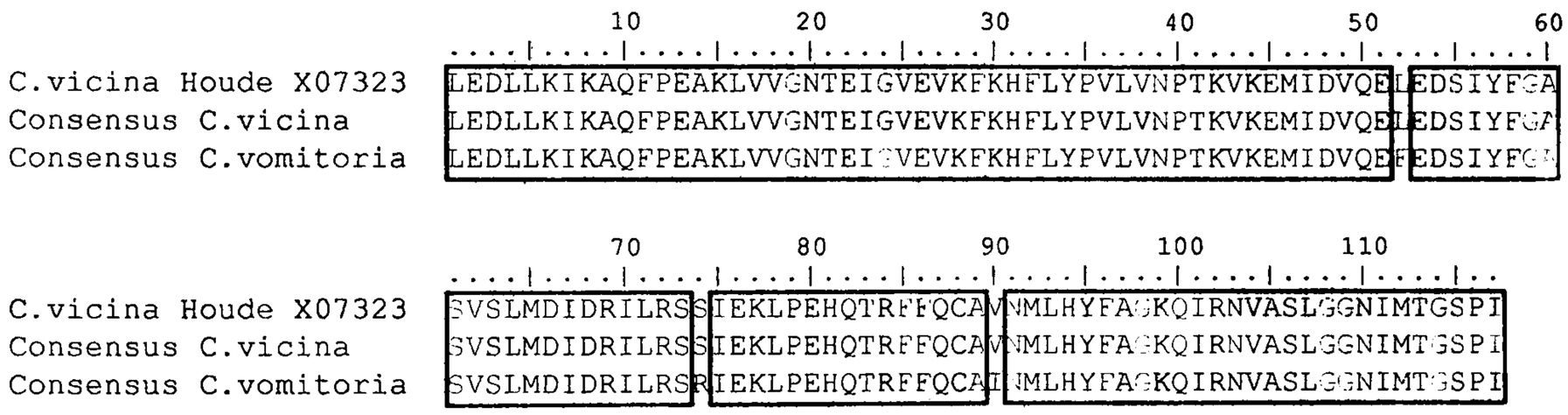


Figure 7.4. Alignment of *C. vicina* and *C. vomitoria* partial XDH amino acid sequence and the *C. vicina* sequence by Houde *et al.* (1989).

7.2.3 Intraspecific variation

After sequencing this partial XDH region it is evident that intraspecific variation exists. This nucleotide variation not only exists between individuals but different alleles also exist within individuals. This is similar to that at the *Eco*RI site (which falls at base number 844 in the sequenced fragment). Figure 7.5 illustrates how within individual variation appears in the raw sequence data. The heterozygote appears as two peaks overlaid at the same position. Occasionally if a DNA sample contains contaminants i.e. if the sample was not cleaned sufficiently post-PCR, then the

chromatogram can have a 'noisy' baseline – low peaks beneath the true peaks. To differentiate between baseline and the heterozygote alleles the peak heights of the potential alleles were compared to surrounding baseline peaks using the BioEdit software. As Figure 7.5 illustrates, the base calling software on the ABI 310 Genetic Analyser cannot differentiate between the heterozygote peaks and so designates the base as the taller peak, in this example 'C'. For this reason chromatograms should always be examined when analysing DNA sequencing data.

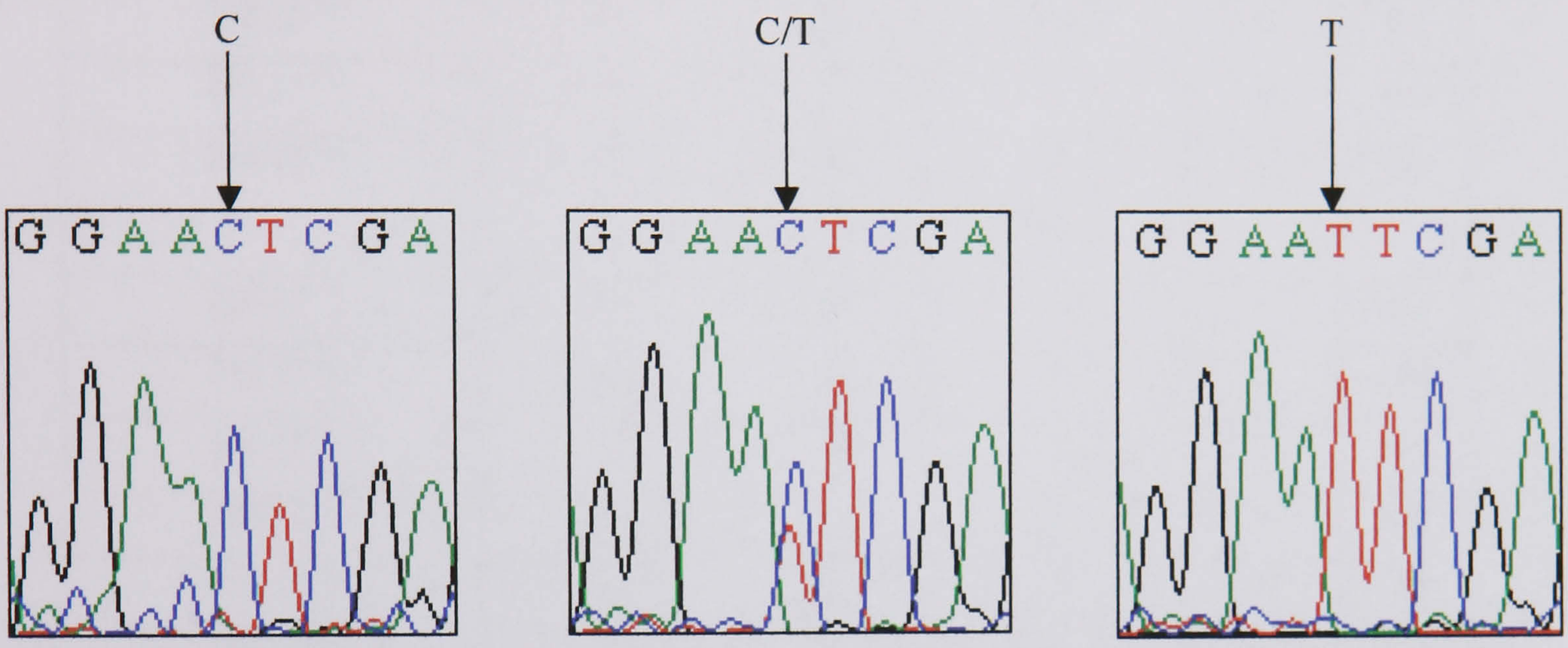


Figure 7.5. Sequencing chromatograms from the XDH DNA sequence to illustrate the appearance of heterozygotes. Normally peaks appear distinct but in the case of two different alleles within the same individual the two peaks overlap as demonstrated in the central picture.

The regions of intraspecific variation in both species are illustrated in Table 7.5. The most common allele, as presented in the consensus sequences, is designated as allele '1' and the alternative alleles '2' or '3'. The amino acids for the different alleles are included, along with the D-values of Grantham (1974).

Table 7.5a. *Calliphora vicina* intraspecific variation within the partial XDH gene region sequenced in this study. D values after Grantham (1974).

Position (Codon Position)	Consensus Nucleotide	Amino Acid	Variation	Amino Acid	D value
690 (3rd)	T	N	C	N	n/a
694 (1st)	G	E	C	Q	29
			A	K	56
708 (3rd)	G	K	T	N	94
711 (3rd)	T	I	C	I	n/a
714 (3rd)	G	K	T	N	94
			C		
726 (3rd)	G	P	A	P	n/a
738 (3rd)	G	L	A	L	n/a
741 (3rd)	T	V	G	V	n/a
750 (3rd)	C	N	T	N	n/a
757 (1st)	A	I	G	V	29
771 (3rd)	T	V	G	V	n/a
783(3rd)	T	H	C	H	n/a
819 (3rd)	T	V	G	V	n/a
844 (1st)	C	L	T	F	22
894 (3rd)	T	D	C	D	n/a
897 (3rd)	G	R	T	S	110
913 (1st)	A	I	T	L	5
957 (3rd)	C	A	T	A	n/a
960 (3rd)	C	V	A	V	n/a
			G		
969 (3rd)	G	L	A	L	n/a
984 (3rd)	T	G	C	G	n/a
			G		
999 (3rd)	T	N	C	N	n/a
1020 (3rd)	T	N	C	N	n/a
1038 (3rd)	C	P	A	P	n/a
1042 (3rd)	T	S	C	P	74

Table 7.5b. ***Calliphora vomitoria*** **intraspecific variation within the partial XDH gene region sequenced in this study. D values after Grantham (1974).**

Position (Codon Position)	Consensus Nucleotide	Amino Acid	Variation	Amino Acid	D value
690 (3^{rd})	C	N	T	N	n/a
708 (3^{rd})	G	K	T	N	94
710 (2^{nd})	T	I	C	T	89
714 (3^{rd})	G	K	T	N	94
735 (3^{rd})	A	K	G	K	n/a
844(1^{st})	T	F	C	L	22
858 (3^{rd})	T	I	A	I	n/a
978 (3^{rd})	C	F	T	F	n/a
991 (1^{st})	A	I	C	L	5
1042 (3^{rd})	T	S	C	P	74

The majority of the intraspecific nucleotide substitutions in *C. vicina* are synonymous. There are four variable residues in common to both species. The D values presented as a measurement of the extent to which the amino acid substitution will affect overall protein structure are all relatively low. The highest is 110 for a potential arginine to serine change in the *C. vicina* samples.

The frequency of this intraspecific variation within each population is presented in Appendix V. The highest level of variation within one individual is 1.97% (7 bases out of 355bp) in *C. vicina* Snaresbrook 3, Wimbledon 9 and Wimbledon 12.

Examples of sequences that show variation have been deposited in GenBank with accession numbers (AY944470-504). For both species there are some alleles that only appear in one population. These are known as private alleles and are highlighted in Table 7.6.

Table 7.6. Private alleles within XDH region of the populations of *C. vicina* and *C.vomitoria*. Allele frequencies are also included.

	Population	Position	Nucleotide	Freq
C.vicina	Cheltenham	694	C	0.125
	Cheltenham	894	C	0.188
	York	757	G	0.200
	Keswick	999	C	0.100
	Keswick	1042	C	0.200
	Great Yarmouth	984	C	0.167
	Great Yarmouth	984	G	0.167
	Snaresbrook	714	T	0.250
	Nonsuch Park	714	C	0.200
	Wimbledon	771	G	0.154
C.vomitoria	York	710	C	1.000
	Keswick	1042	C	0.500
	Commercial	708	T	0.143
	Commercial	714	T	0.036
	Commercial	844	C	0.143
	Commercial	991	C	0.036

Initially the populations were assessed as to whether they were in Hardy-Weinberg equilibrium. This was conducted using the exact test, as described by Guo and Thompson (1992). Chi squared values (χ^2) are calculated for those populations showing heterozygosity within this gene region.

Table 7.7. Chi squared and probability values of Hardy-Weinberg exact test for the populations of *C. vicina* and *C. vomitoria*. n/c – not calculated. Values calculated based upon the XDH amplicon sequenced in this study

C. vicina			*C. vomitoria*		
Population	X^2	p value	Population	X^2	p value
Cheltenham	28.81	0.05*	York	n/c	
Waterloo	n/c		Keswick	13.72	0.03*
Greenwich	n/c		Petts Wood	n/c	
Stanmore	n/c		Nonsuch Park	n/c	
York	21.92	0.04*	Box Hill	23.45	0.00*
Nottingham	13.16	0.11	Commercial	20.63	0.01*
Keswick	21.57	0.04*	Mitcham Junction	0	1.00
Derby	n/c		Hampstead Heath	n/c	
Great Yarmouth	9.61	0.29	Wimbledon Common	20.43	0.00*
Pontypridd	2.1	0.70	Cheltenham	n/c	
Snaresbrook	5.08	0.96	East Sheen	n/c	
Mitcham Junction	25.29	0.00*	Stanmore	n/c	
Nonsuch Park	24.59	0.14	Total	78.22	0.00*
Wimbledon	93.45	0.00*			
Total	245.68	0.00*			

The total χ^2 values for both species indicate that they are not in Hardy-Weinberg equilibrium as they are significant at the 5% level. Individually, some of the *C. vicina* populations do not depart from the expected heterozygosity for populations in Hardy-Weinberg equilibrium. The *C. vomitoria* Mitcham Junction population appears to be in Hardy-Weinberg equilibrium too.

An AMOVA was conducted to assess the variation between and within populations (Table 7.8 a and b).

Table 7.8a. AMOVA based upon XDH *C. vicina* intraspecies variation.

Source of Variation	df	SSq	MSq	Est. Var.	% Variation	F_{ST}	Prob
Between Populations	12	57.671	4.806	0.357	22%	0.221	0.010
Within Populations	119	150.246	1.263	1.263	78%		
Total	131	207.917					

Table 7.8b. AMOVA based upon XDH *C. vomitoria* intraspecies variation.

Source of Variation	df	SSq	MSq	Est. Var.	% Variation	F_{ST}	Prob
Between Populations	10	25.070	2.507	0.174	33%	0.330	0.010
Within Populations	129	45.366	0.352	0.352	67%		
Total	139	70.436					

The null hypothesis in both cases can be rejected; the between population variation is significantly greater than would be expected if the population structure did not affect the genetic structure of the species. From the allele frequencies in Appendix V, Wright's F-statistics and the equivalent Weir and Cockerham parameters could be calculated to assess population structure of both species (Table 7.9). The AMOVA also gave an estimate for F_{ST}.

Table 7.9. F-statistics for *C. vicina* and *C. vomitoria* populations. F-statistics calculated after the formulae of Wright and from the AMOVA.

	Wright				Weir and Cockerham				AMOVA	
	F_{IS}	F_{IT}	F_{ST}	N_m	F_{IS}	F_{IT}	F_{ST}	N_m	F_{ST}	N_m
C. vicina Mean	0.390	0.509	0.231	0.833	0.572	0.640	0.160	1.313	0.221	0.881
C. vomitoria Mean	0.510	0.608	0.318	0.536	0.639	0.749	0.305	0.570	0.330	0.508

To assess pairwise differences between populations, the average genetic distance between them was calculated using the Tamura-Nei correction factor (data presented in Appendix V). The *C. vicina* and *C. vomitoria* populations were considered separately. The within population differences were calculated by the same method. The relationship between population genetic and actual geographic distance is demonstrated for both species graphically (Figures 7.6 and 7.7). A Mantel test was conducted on the geographic distance and genetic distance matrices (Appendix V) and is presented in Table 7.10.

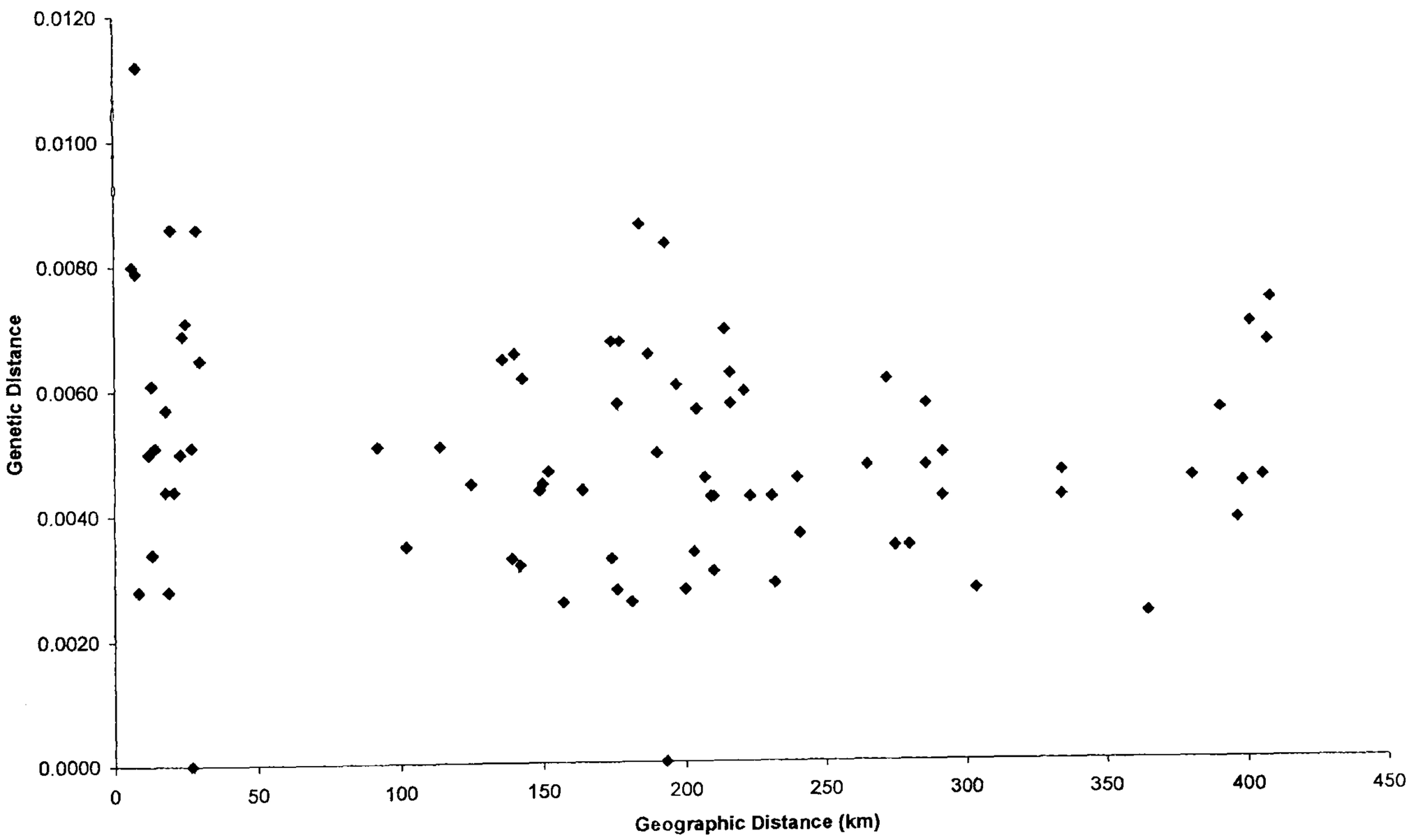


Figure 7.6. Pairwise genetic distances versus geographic distances for *C. vicina* populations.

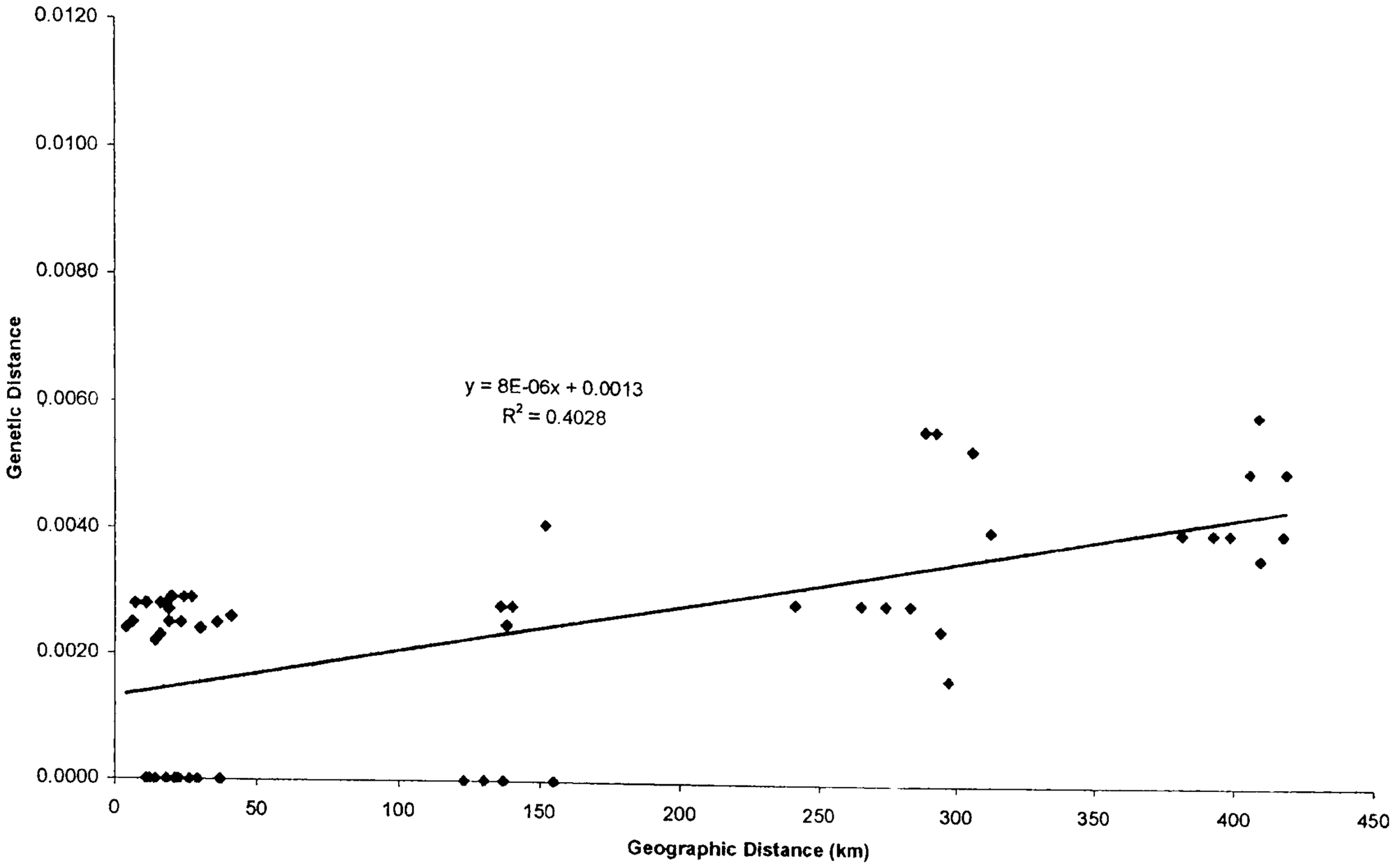


Figure 7.7. Pairwise genetic distances versus geographic distances for *C. vomitoria* populations.

Table 7.10. Mantel correlation coefficients (Rxy) between *C. vicina* and *C. vomitoria* population genetic and geographic distances. SSx – variance of geographic distance. Ssy – variance of genetic distance. SPxy – covariance of geographic and genetic distance.

	SSx	SSy	SPxy	Rxy	p value
C.vicina	1204696.750	0.0003	-2.425	-0.126	0.726
C.vomitoria	1178546.875	0.0002	8.908	0.635	0.017

The graph in Figures 7.6 demonstrates that there is no relationship between the genetic and geographic distance between *C. vicina* populations. A trendline can not be fitted to this data as R^2 values are low. The Mantel correlation statistic also indicates that there is no relationship between genetic and geographic distance for *C. vicina* populations.

There is a significant positive relationship between genetic distance and geographic distance in the *C. vomitoria* populations (Rxy=0.635 $p<0.05$). Genetic distance increases with geographic distance between populations. The data are a better fit to this line than the *C. vicina* data as the R^2 value is much greater (0.4028).

The genetic distance between the populations was further demonstrated by the neighbour-joining trees produced for *C. vicina* and *C. vomitoria* populations (Figure 7.8 and 7.9). Also included in the *C. vicina* tree are the other *C. vicina* XDH sequences deposited in GenBank. The GenBank entries give no indication of their population location. According to the literature from the respective authors it was assumed that all the sequences (X07323, M30316, M18423 and X17106) came from *C. vicina* caught in France.

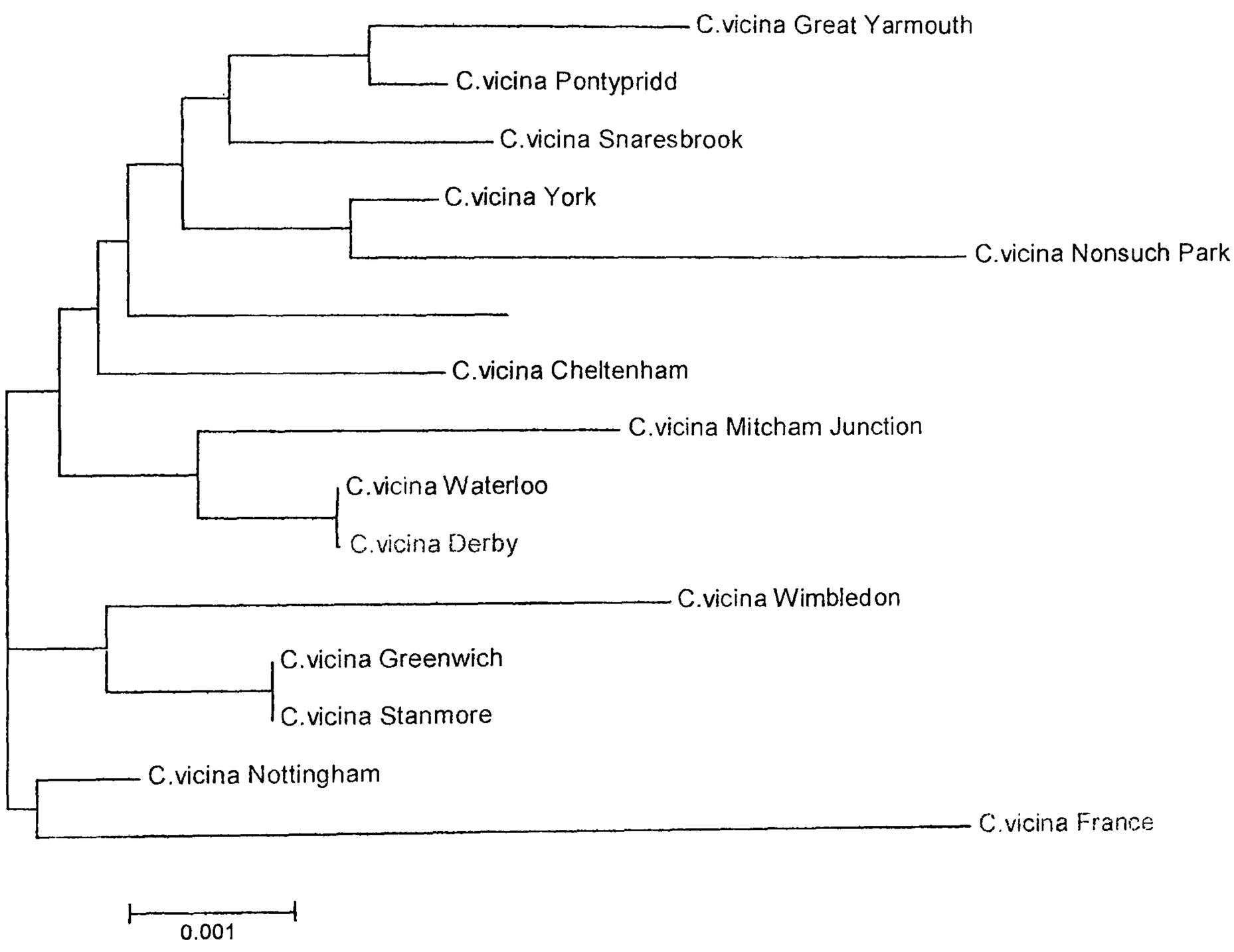


Figure 7.8. Neighbour joining tree constructed from Tamura-Nei genetic distances between *C. vicina* populations.

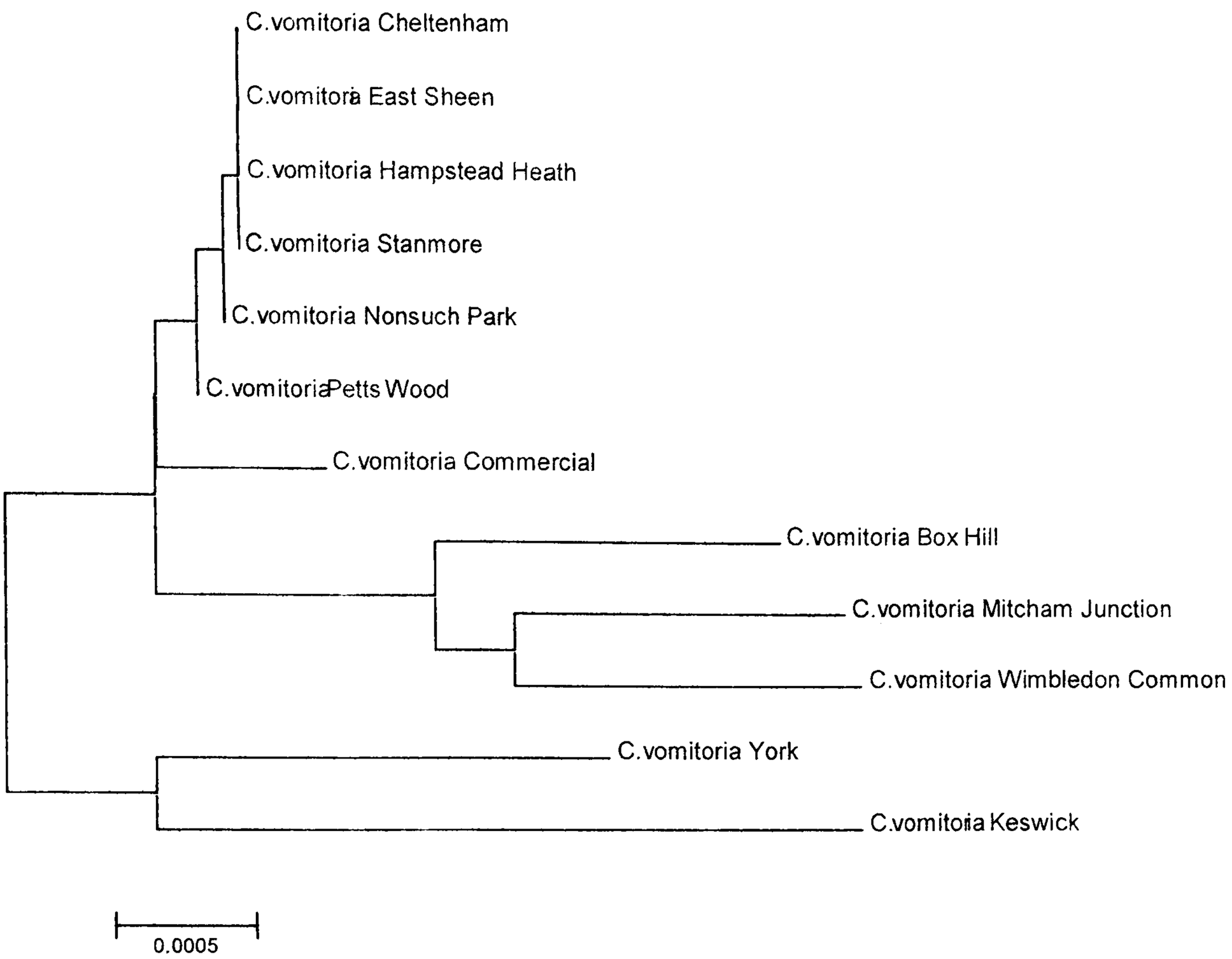


Figure 7.9. Neighbour joining tree constructed from Tamura-Nei genetic distances between *C. vomitoria* populations.

Neighbour-joining trees were also constructed of all the samples sequenced in this study. This was to assess whether the within population variation (values presented in Appendix V) alters the groupings evident in the trees established with the average population genetic distances.

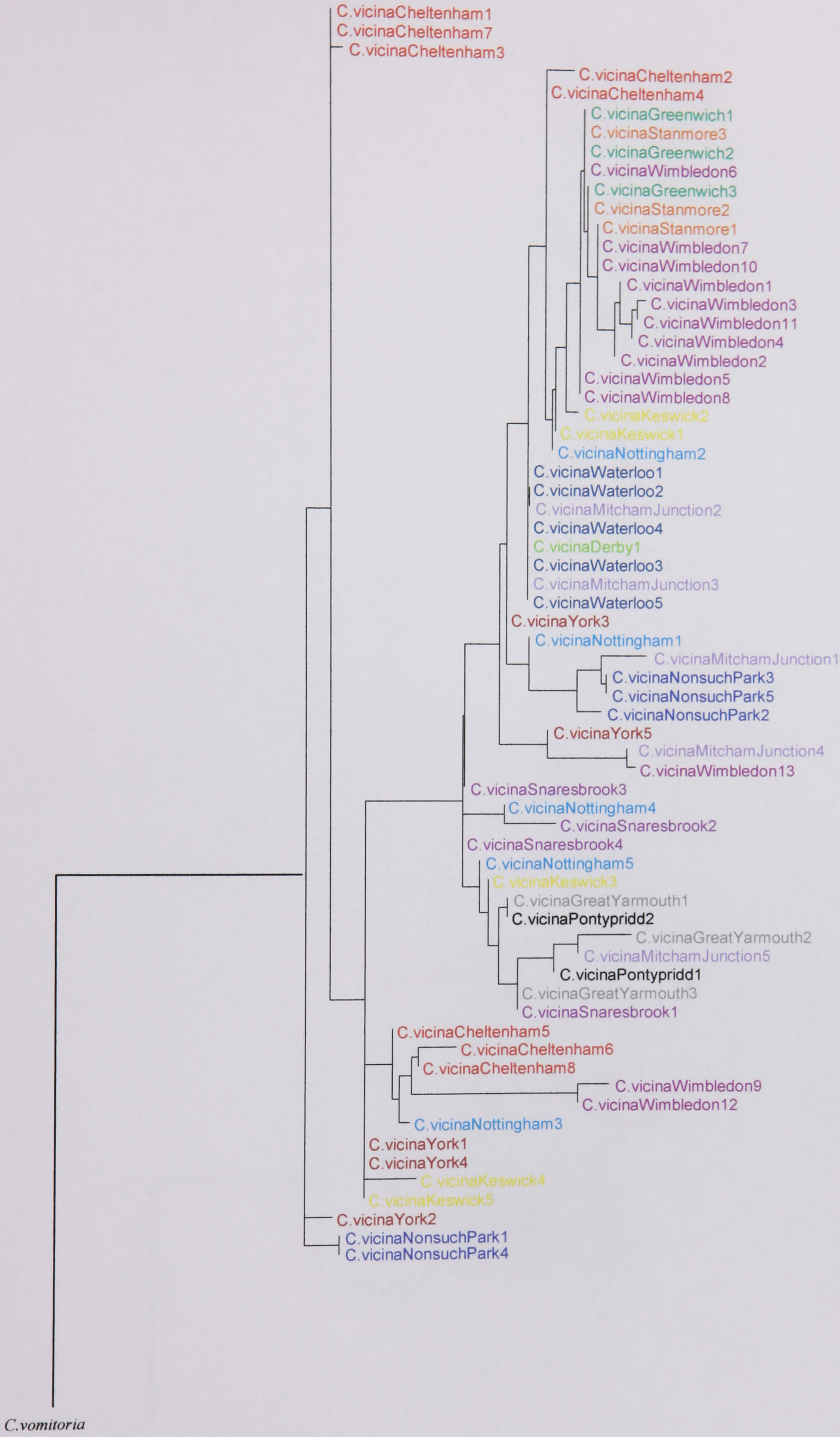


Figure 7.10. Neighbour joining tree based on an alignment of partial XDH region. *Calliphora vomitoria* topology is condensed in order to fit all *C. vicina* onto one page.

Figure 7.10. Neighbour joining tree based on an alignment of partial XDH region. *Calliphora vicina* topology is condensed in order to fit all *C. vomitoria* onto one page.

To assess whether these XDH markers can be used to assign an unknown sample to a population, the 'population assignment' test based upon Paetkau *et al.* (1995, 2004) using GenAlEx software was conducted (Appendix V).

For the *C. vicina* populations, the test assigned 47.8% of samples to their correct population, 17.9% to several populations including the correct one and 34.3% to the wrong population. For *C. vomitoria* the test assigned 25.4% of samples to the correct population, 40.8% to several populations including the correct one and 33.8% to the wrong population.

7.3 Discussion

7.3.1 *Eco*RI Restriction Enzyme Polymorphism

Rocher-Chambonnet *et al.* (1987) and Houde *et al.* (1989) noted the differences in fragment pattern of the XDH exon 2 *C. vicina* gene region after digestion with the *Eco*RI enzyme. With the XDH amplicons designed and used in this experiment the polymorphism is evident as either one large band on an agarose gel or two smaller bands. After sequencing the reason for the different fragment patterns after digestion became apparent. It was due to a C<>T nucleotide transition at one of the two enzyme cut sites. When assessing the fragment patterns, the majority of *C. vicina* samples in this study were Genotype A (one large band) whereas the majority of *C.vomitoria* were Genotype B (two smaller bands). Although there is a clear difference between the two species, this region of the XDH gene alone cannot be used to distinguish the two species as both contain intraspecific variation.

The present study has shown that variation exists within the *C. vomitoria* species as well as within *C. vicina* as noted by Rocher-Chambonnet *et al.* (1987). Rocher-Chambonnet *et al.* and Houde *et al.* are based in France and it is assumed that the specimens used were local to them. This study has utilised English and Welsh *C.vicina* specimens and has indicated that the polymorphism is also evident in these samples along with the *C. vomitoria* samples included.

The *Eco*RI variation is present in four of the 14 *C. vicina* populations and one of the *C. vomitoria* populations included. Whilst the number of individuals demonstrating variation is relatively low when compared to the total number of samples (8% for *C. vicina* and 5% for *C. vomitoria*), the polymorphic populations have it at a comparably high level. *Calliphora vicina* Cheltenham population has 27% individuals containing the alternate nucleotide. Keswick has a 40% level, Nottingham has a 20% level and Waterloo demonstrates a 12.5% intrapopulation variation level.

It could be argued that variation could actually be present in other populations and would have been evident, had a greater sample number been used. This is true for such populations as Derby and Pontypridd that are represented by one and two individuals respectively, due to limited samples trapped at these locations. If the average level of variant individuals present in the polymorphic *C. vicina* populations was actually present throughout all populations, in theory a minimum sample size of 4 is required for one individual examined to demonstrate variation (average variation 24.9% 1/0.25 = 4). Derby, Great Yarmouth and Pontypridd sample sets had ≤ 4 individuals. The AB/B genotypes could actually be present in these populations without being evident in this study.

Conversely, inclusion of larger population sample groups, especially the *C. vicina* Wimbledon population of 26 individuals, with none indicating the B allele implies that this polymorphism does vary population to population and is not simply a case of chance sampling variation.

Within the *C. vomitoria* samples examined in this study only the commercially bought individuals indicated any variation (~21%). Similar to *C. vicina* it appears that this polymorphism is linked to population, as it is not present in the other *C. vomitoria* populations examined here. As with *C. vicina*, the Cheltenham and York population samples used in this work could be too small to be representative of the true Cheltenham and York *C. vomitoria* populations. But if the A allele in *C. vomitoria* is widespread across England then it should have been evident in more samples examined in this work.

As noted previously, the majority of *C. vicina* are of Genotype A and *C. vomitoria* are Genotype B. After analysis of the DNA sequences and amino acid sequences it has been shown that Genotype A is has a C nucleotide at the enzyme restriction site which translates as a leucine amino acid and Genotype B has a T nucleotide at the *Eco*RI site which translates to a phenylalanine residue. The D value, as described by Grantham (1974), between these two amino acids is 22. This is classified as a conservative

amino acid substitution according to Li *et al.* (1985). It is unlikely that either amino acid offers any selective advantage to the final protein structure.

7.3.2 *C. vicina* and *C. vomitoria* XDH Sequence Comparison

After sequencing of an internal region of the XDH exon 2, consensus DNA sequences for *C. vicina* and *C. vomitoria* were produced and deposited in GenBank. Until this study there were no *C. vomitoria* XDH sequences in GenBank. The nucleotide percentage frequencies for these sequences are different to those obtained by Rodriguez-Trelles *et al.* (1999) for *Drosophila* species. The *Drosophila* species averages show a definite tendency to G in the 1st position within the codon whereas in *Calliphora* there is a greater amount of A in the 1st position although G is the next frequent. In the 2nd position the least common nucleotide is G in both genera but in *Drosophila* A features more frequently whereas in *Calliphora* T is the most common. In the 3rd position T is the most common in *C. vicina* and the average *Drosophila* species compared to A in *C. vomitoria*. The greatest differences are evident in the total nucleotide percentages across the XDH region. For the average *Drosophila* species the nucleotide percentages are about 25% as might be expected. For the *Calliphora* species however, nucleotides are more biased towards T and A nucleotides. The levels of A+T nucleotides within this nuclear gene are not as great as found in the mitochondrial COI gene region.

7.3.3 Interspecific variation

The consensus alignment showed 22 nucleotide substitutions between species. There is a 12:10 ratio of transitions: transversions. As the majority of differences are located at the third base position within the codon, therefore, even with the relatively high level of transversions, 19 out of 22 variant nucleotides are synonymous. The three non-synonymous changes only occur in this gene region when variation occurs in the first position within the codon. This includes the Genotype A/B at position 844 as discussed. The other two non-synonymous differences between the species are at

nucleotide positions 910 (serine in *C. vicina* and arginine in *C. vomitoria*) and 958 (valine in *C. vicina* and leucine in *C. vomitoria*). Using the amino acid difference figures produced by Grantham (1974) the arginine/serine change is classed as moderately radical (D=110), due to serine being uncharged and arginine positively charged. Even though this is a moderately radical change, it cannot interfere negatively with protein function as it is still a functional gene in both species. The valine/leucine change is classed a conservative amino acid change (D=29) as both are nonpolar amino acids, using the class boundaries and definitions of Li *et al.* (1985).

According to the 3D structure of the XDH protein these three differences are located in a coil structural element (substitution 910) and an internal and external α helix (958 and 844 respectively). This does not agree with Houde *et al.* (1989), who concluded that most differences would be located to the exterior of the protein, where structural restraints would be less. They did however note that replacements were more likely to occur in α helices than β sheets.

When comparing *D. melanogaster* and *C. vicina* XDH regions Houde *et al.* (1989) noted a high level of synonymous interspecific base differences, which was also observed between *C. vicina* and *C. vomitoria* in this work. They found that the level of nucleotide differences (1272 out of 4008bp ~ 32%) was higher than the difference between amino acid sequences (24.5%). Previous to this work and that of Houde *et al.* (1989), Rocher-Chambonnet *et al.* (1987) noted that the divergence of *C. vicina* and *Drosophila* has been restricted by minimal change to the overall protein structure.

However the XDH protein final structure must allow some change as Buchanan and Johnson (1983) observed the presence of different XDH allozymes in *Drosophila* species when separated on a gel. As discussed in Section 3.1.3, allozyme separation by electrophoresis indicates differences in a protein's amino acid sequence.

7.3.4 Comparison of Inter and Intra-specific variation

The *Eco*RI restriction digest alluded to a level of intraspecific variation within this gene region that became evident after DNA sequencing. Examination of this intraspecific variation indicated the limitation of XDH as a *Calliphora* species marker. Whilst XDH has five more interspecific differences than the COI region identified in Chapter 6, there is a higher level of intraspecific variation in XDH that could potentially cause misidentification. On comparison of all samples in this study, 10 out of the 22 'interspecific' differences between the *C. vicina* and *C. vomitoria* consensus sequences show the nucleotide of one species within the intraspecific variation of the other. E.g. at nucleotide position 726, the *C. vicina* consensus sequence has a G nucleotide and the *C. vomitoria* consensus has an A. Closer examination of the *C.vicina* sample sequences show four *C. vicina* specimens with an A and seven with R (both G and A within the same individual). The GenBank *C. vicina* sample X17106 also shows an A at this nucleotide position. At position 873, all the *C. vicina* specimens sequenced in this work have a C nucleotide and all *C. vomitoria* have an A. However inclusion of the GenBank samples indicates that this is not an interspecific substitution as one of the *C. vicina* samples has an A at this position (X17106). Sequence X17106 often differs from the other sequences entered in GenBank. In most positions the other three sequences will be identical to the majority of samples in this study, whereas X17106 contains the nucleotides represented in a minority of samples. As discussed in Chapter 6, it is important to include the largest sample number possible so that this type of variation is not overlooked.

Two other nucleotide positions (711 and 819) show both interspecific and intraspecific differences but could still be used as molecular markers for species differentiation. At 711, all *C. vomitoria* have an A nucleotide compared to T, C or Y (T and C in one individual) in *C. vicina*. Similarly, at position 819, all *C. vomitoria* have an A nucleotide whereas *C. vicina* either have a T (the majority) or a G base (3 individuals).

7.3.5 Variation between and within Populations

The XDH gene region shows variation between both *C. vicina* and *C. vomitoria* populations. It was demonstrated that, like the interspecific variation, most nucleotide substitutions would be synonymous. Non-synonymous changes had low D values apart from a potential arginine to serine change in some *C. vicina* samples. All these samples were heterozygotes and therefore the change might not even be evident in the final protein. It is unlikely that any of these non-synonymous changes offers any great selective advantage in the final protein, as most are conservative changes to the amino acid sequence.

After examining the allele frequencies of the populations, it is evident there are no private alleles present in all samples within a population (apart for *C. vomitoria* York population, which only was based upon one sample and therefore cannot be assumed as representative for the whole population). This implies there is not one specific nucleotide polymorphism in the partial XDH region sequenced in this work that can be used to identify a population. The nucleotide variant sites need to be used in conjunction with each other to differentiate the populations.

The blowfly populations were tested to examine whether they are in Hardy-Weinberg equilibrium. The significance of the test statistic for the total samples implies both species deviate from the equilibrium. The high F_{ST} statistic implies there is genetic differentiation between populations. The positive F_{IS} values imply mating is not random, as assumed in the Hardy-Weinberg calculations; instead inbreeding within populations appears to be occurring. It should be noted that some of the *C. vicina* populations individually were not significantly different to a population mating randomly, even though the overall result indicated that they were. The results indicated that there are few migrants between the *Calliphora* populations and therefore gene flow will be low between populations. However, the mathematical formula for calculating N_m from F_{ST} values only applies if the assumptions made for the Island model are realised. These assumptions (e.g. no selection, no mutation and particularly that the populations are in drift-migration equilibrium) are rarely true in

natural systems (Whitlock and McCauley 1999) and therefore migration between blowfly populations should be examined further before conclusions are made as to the numbers of migrants.

Average genetic distances were calculated for populations within each species. When these are compared to the interspecific value (0.0653) it can be seen that the genetic distances between the populations are very small. Most are about ten-fold smaller that the difference between *C. vicina* and *C. vomitoria* consensus sequences, which differ by 22 nucleotide residues. The genetic difference between *C. vicina* and *D. melanogaster* is 0.4551 (111 nucleotides different). This indicates the difference between two dipteran species from different families. Therefore whilst the differences between populations are significant, it should be stressed that the populations do not vary by many nucleotide substitutions.

When the genetic distances are compared to geographic distance, they showed no correlation for *C. vicina* populations but were significantly positively correlated for *C. vomitoria* populations. This indicated that there is some isolation by distance for this species – a property of the stepping-stones model of migration – gene flow occurs between neighbouring populations.

This concurred with the neighbour joining trees constructed from the average genetic distances. The groupings for *C. vicina* on the tree are not related to geographic location. For example, the Pontypridd population samples as a whole are most similar to Great Yarmouth samples that were caught 365km away. The tree for *C. vomitoria* shows an approximate North/South England separation with York and Keswick separate from the other populations. However, only one *C. vomitoria* York sample was included (due to trap limitations) which may have produced the appearance of a North/South genetic separation and a significant correlation between genetic and geographic distance.

Interestingly, the 7 populations from which samples were included from both species do not show similar groupings. In the *C. vicina* tree, York, Nonsuch Park, Keswick and Cheltenham are distinct from Mitcham Junction, which in turn is separated from Wimbledon and Stanmore. For *C. vomitoria,* Cheltenham and Stanmore are on the same branch of the tree, separate from Mitcham Junction and Wimbledon and also York and Keswick, which are on two separate branches of the tree.

Principal component analysis was conducted on the data (graphs not presented) and these showed exactly the same groupings of populations as the neighbour joining trees. Whilst the production of trees is a more conventional method of examining the relationships of separate populations, principle component analysis is another method that could be employed, as utilised by Hwang (2004).

Likewise pairwise F_{ST} and N_m values were also calculated between all populations (not presented) that showed greater gene flow between the population groupings indicated by the neighbour joining trees.

A neighbour joining tree of all the samples in this study was produced. Unlike the phylogenetic analysis of the COI gene region, this XDH tree was not rooted on another species, as few XDH genes have been sequenced. A tree (not shown) was rooted upon a *D. melanogaster* sequence (AY279338) and as the level of variation within the *Calliphora* species was relatively low compared to between them and the *D. melanogaster* sequence the tree was uninformative – the *Calliphora* species were clustered together and it did not give any intraspecific resolution.

The tree of total samples shows some differences with the neighbour joining trees drawn from average genetic values. This might have been expected due to the fairly high intrapopulation genetic differences calculated for some populations. The *C.vicina* tree (Figure 7.8) showed Nottingham population grouped with the French samples distinct from the other English populations. When broken down into separate samples (Figure 7.10), Nottingham samples are spread through the tree topology. The same is true for the Keswick, Cheltenham and York samples. In the tree of averaged distances these three populations were on branches close to each other. The separated tree shows the samples are spread throughout the tree. Cheltenham samples consist of three distinct groups. One distant to all other samples, with a haplotype of nucleotide 726 ~ R; 741 ~ G; 783 ~ Y; 844 ~ T/Y; 894 ~ Y; 960 ~ S. One grouped with Wimbledon and Nottingham (Nucleotide 690 ~ Y; 694 ~ A; 741 ~ Y; 783 ~ C/Y) and the third in the larger grouping of Greenwich, Stanmore, Wimbledon and Keswick

(nucleotide 960 ~ R). The majority of Wimbledon samples are still grouped with Stanmore and Greenwich samples as they appeared in the averaged distances tree (these all have sequences similar to the established consensus sequence apart from a G nucleotide at 960).

The major difference for the *C. vomitoria* samples is that the Keswick and York grouping in the averaged tree (Figure 7.9) does not appear in this tree. In this phylogram, the York sample appears alongside the Petts Wood samples. One Keswick sample is located with Box Hill, Wimbledon Common and Mitcham Junction group and the others are located alongside Nonsuch Park samples. The genetic distance calculated within the Keswick population was 0.006, which is greater than many of the between population distances.

These markers within the XDH region exemplify, as suggested previously, that barriers such as rivers do not stop the dispersal of *Calliphora* adults (MacLeod and Donnelly 1958) as the London groups shown in this study span the River Thames.

The population assignment analysis assigned two thirds of samples to the correct population (or the correct population grouping). All samples within the *C. vicina* Nonsuch Park population and the *C. vomitoria* Mitcham Junction were all assigned to the correct population. The loci markers used in this study are all that is required to identify these samples. Conversely, one third of both *C. vicina* and *C. vomitoria* samples were assigned to incorrect populations. This number is relatively high and highlights the requirement to find other genetic markers either within the XDH gene or elsewhere to aid in differentiating between populations of blowfly. GenAlEx was used to conduct this test after the work of Paetkau *et al.* (1995, 2004). Cornuet *et al.* (1999) indicated that methods involving the use of log likelihood measurements (as the Paetkau *et al.* method does) were more accurate than distance based models. However, they concluded that of the likelihood methods the most efficient was a Bayesian model. Using their software program GeneClass 2.0 (Cornuet *et al.* 2005), the data from this study were subjected to several methods of population assignment and indicated that for these data, the log likelihood method of Paetkau *et al.* assigned more populations correctly than the Bayesian models. The results did concur with

Cornuet *et al.* (1999), that the distance methods produced the least accurate results. Only the Paetkau *et al.* log likelihood results are presented in this work.

In summary, although no nucleotide polymorphisms were found in this section of the XDH gene that were distinct to particular *C. vicina* and *C. vomitoria* populations, this work indicates that English *C. vicina* and *C. vomitoria* populations do show genetic differences and further regions should be examined to allow the characterisation of differentiation of UK populations.

Chapter 8

Use of molecular markers with different specimen types

As described in Section 1.4.8, identification of immature stages using morphological characteristics is more difficult than that of adults. Whilst adult flies can be collected from a crime scene, it will most likely be the immature forms (eggs, larval stages and pupae) that are obtained from the vicinity of a corpse. Indeed it is these specimens that will provide more information to estimate minimum PMI. The benefit of using a DNA marker system for identification is that molecular characteristics should be independent of age of insect. It is unlikely that the marker will be altered by mutation as an individual ages. As Chapter 6 has shown there was no difference between the COI DNA sequences for all adults and all immatures within each species. The work on COI also illustrated that DNA could be extracted from wandering larvae in a suitable form for PCR and subsequent analysis. It was now pertinent to show that the same is true for other *Calliphora* lifestages. Vincent *et al.* (2000) demonstrated using a phenol/chloroform extraction method that DNA could be obtained from the pupal stage.

Not all entomological evidence at a crime scene will be alive when discovered and collected. If insects have experienced extremes of temperature or high levels of carbon dioxide they will die in the vicinity of the corpse (Staerkeby 2001). Greenberg and Tantawi (1993) showed that all experimental *C. vomitoria* specimens at 35°C died in the wandering stage. Reiter (1984) indicated that *C. vomitoria* larvae did not pupate successfully at a constant 30°C and eventually died.

If a corpse is close to water and larvae are unable to escape, submersion of immatures will cause death in most cases, although a low percentage of *C. vomitoria* wandering larvae have been observed to survive submersion in water at room temperature for four days (personal observation) and Singh and Greenberg (1994) demonstrated nearly 100% survival of late pupal stage after 24h under water. They did however show near complete mortality after submersion of pupae for 2 or more days. It is likely that if insects become submerged for long periods, dead insects will be recovered from the scene.

Alternatively, if a body were set alight post-mortem (after insect invasion) this would also burn the insects associated with the corpse. Anderson actually found live insects after simulated arson fires on pig corpses (Anderson 2004). Enough evidence was found to allow calculation of minimum PMI and therefore entomologists still need to be able to identify specimens after burning.

Any dead insects in the environment will undergo decomposition until discovery. This change in state might affect the morphology and not allow identification. It has not been established whether decomposition or burning will also affect success of molecular analysis.

The presence of dead insects at a crime scene means that the investigator cannot gain the best estimate for PMI, as it is difficult to ascertain when the insects died. However, the presence of certain species will provide information, especially if a species is outside of its geographic range. Also if the stage reached when they were killed can be established (for instance if the immatures are intact this can be done by length) this will provide a minimum PMI. Therefore accurate identification of dead insects is important in the absence of live specimens.

Other blowfly-associated evidence can remain at a scene of crime. If any Calliphoridae present reach the adult stage, then empty pupal cases will be left in the surrounding area of the corpse after adult emergence. Presence of pupal cases will provide a minimum PMI, even though it will be a less good estimate than if discovery of the body occurs before adult emergence. Pupal cases are often found long after the corpse has decomposed and some have even been found in good condition after thousands of years (Erzinçlioğlu 1985). Capelli *et al.* (2003) in a comparison of forensic and ancient DNA analysis techniques noted that amplifiable DNA could survive up to 100,000 years, so pupal cases should still be identifiable after many years, by DNA analysis. Vincent *et al.* (2000) also included empty puparia in their study and adapted the phenol/chloroform protocol so that DNA was extracted in a smaller volume of buffer. They obtained enough DNA "for several PCR reactions". Of course, if decomposition of a corpse is that advanced that adult Calliphoridae have emerged, the presence of other forensically interesting invertebrates will aid in a better estimate of PMI.

It is best practice when carrying out molecular techniques on insect evidence to keep vouchers for future identification/reference purposes and not to use the whole sample for DNA extraction. It is therefore important to know which body parts of an adult fly are required for adequate DNA retrieval. Wallman and Donnellan (2001) advise the use of adult thoraxes for extraction as this allows the head and abdomen to be kept as vouchers. Other researchers suggest the use of leg muscle for DNA extraction (Stevens and Wall 2001). This of course is based upon whole specimens being available. It is possible that only partial samples might be presented and therefore it is important to know whether DNA retrieval is viable from these insect fragments.

The next part of work would ascertain whether the type of entomological sample affects DNA isolation and identification.

8.1 Materials and Methods

8.1.1 Sample Preparation

The following samples were prepared for DNA extraction and subsequent PCR amplification.

8.1.1.1 Lifecycle Stage Samples

Eggs from both species were harvested and incubated at 20°C as described in Section 2.3. As the lifecycle progressed, specimens were taken at specific stages and killed by placing at –20 or -70° C (see Table 8.1 for stages).

8.1.1.2 Adult Body Parts

Adult flies were cut with a sterile scalpel to provide the samples in Table 8.1. Figure 8.1 illustrates the thorax and femur of adult *C. vicina* and *C. vomitoria*.

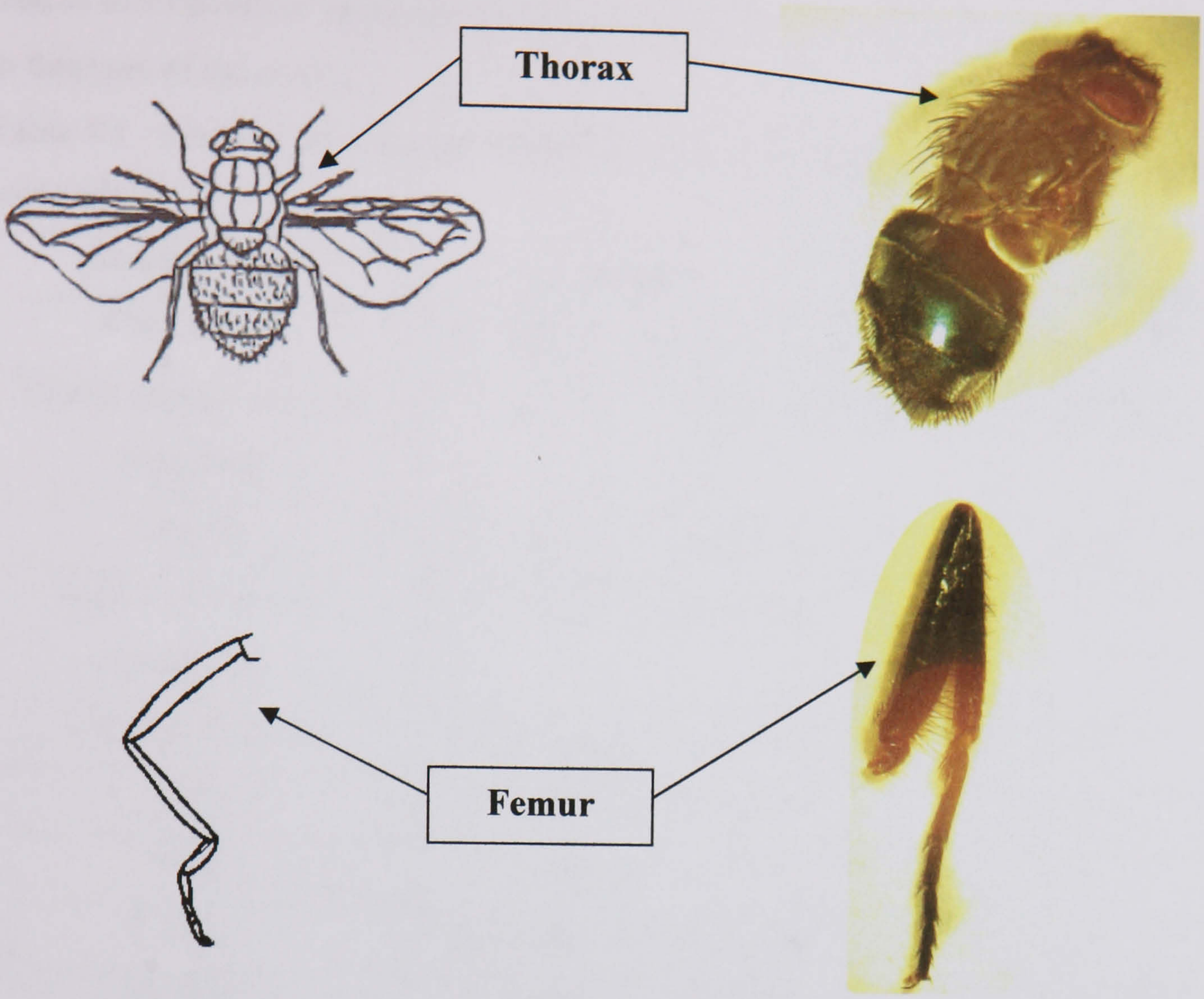


Figure 8.1. Diagrams illustrating the position of the thorax and femur on *Calliphora* adults. Diagrams drawn from Erzinçlioğlu (1996) and Smith (1986). Photographs taken of *C. vicina* adult with wings and legs removed.

8.1.1.3 Burnt and Submerged/Decomposing Samples

Larvae in the wandering stage were placed in aluminium foil along with strips of paper that were set alight. Flames were left until they naturally extinguished. Due to positioning within the foil, maggots received differing amounts of charring but all were killed by the extreme heat. Photographs were taken to show extent of damage.

Other larvae were submerged in water at room temperature for four days. Some were then placed in wet compost in containers described in Section 2.3.1 and placed in a 20°C incubator – this was to recreate decomposition in damp earth under moist environmental conditions.

Others were placed in containers without the compost and put in the same incubator – this allowed the dead larvae to decompose under dry conditions. Both were left in the incubator for four days. Photographs were taken to show features of insects before DNA extraction.

Due to availability of specimens only commercially purchased *C. vomitoria* were used in this part of the study.

Table 8.1. Number of samples of each type for both species used for DNA extraction comparison.

Sample Type	Species	Number of Samples
Whole Adult	*C. vicina ; C. vomitoria*	3 ; 2
Thorax (wings and legs removed)	*C. vicina ; C. vomitoria*	2 ; 2
Leg (1)	*C. vicina ; C. vomitoria*	2 ; 2
Legs (6 from one specimen)	*C. vicina ; C. vomitoria*	2 ; 2
Leg Part (femur)	*C. vicina ; C. vomitoria*	2 ; 2
Wing	*C. vicina ; C. vomitoria*	2 ; 2
Egg	*C. vicina ; C. vomitoria*	1 ; 1
8 Eggs	*C. vicina ; C. vomitoria*	1 ; 1
Larval 1	*C. vicina ; C. vomitoria*	1 ; 1
Larval 1 x 6	*C. vicina ; C. vomitoria*	1 ; 1
Larval 2	*C. vicina ; C. vomitoria*	2 ; 2
Larval 3 (feeding)	*C. vicina ; C. vomitoria*	2 ; 2
Larval 3(wandering)	*C. vicina ; C. vomitoria*	2 ; 2
Pupal	*C. vicina ; C. vomitoria*	2 ; 2
Empty pupal case	*C. vicina ; C. vomitoria*	3 ; 2
Burnt Larval 3 Larvae	*C. vomitoria*	3
Decomposing Larval 3 Larvae	*C. vomitoria*	6

8.1.2 DNA Extraction

All samples were extracted using QIAamp spin columns (QIAGEN) as described in Section 5.2.

8.1.3 PicoGreen Quantification

A PicoGreen assay was set up as described in Section 5.2.5. DNA standards (20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 ng/µl) were included on the 96-well plate. Ten

microlitres of each sample was duplicated on the plate. Plates were read on a Cytofluor 4000 Plate reader (PerSeptive Biosystems).

Using the standards, calibration curves were created in Excel (Microsoft®). From these the concentration of the samples could be established.

8.1.4 DNA Amplification (PCR)

8.1.4.1 COI

The partial COI region was amplified in these samples as described in Section 6.1.2. The volume of template DNA added to each PCR reaction was determined after the PicoGreen quantification, so that between 10-50ng of DNA was added to each reaction. For samples of low DNA concentrations (e.g. wing <0.3 ng/µl and empty puparia samples <1.5 ng/µl) no water was added to the PCR reaction. Reactions consisted of DNA template and water (10.5µl), primers (1µl of each primer (10µM)) and RedTaq™ (12.5µl). Thermal cycling was as Section 6.1.2.

8.1.4.2 XDH

The XDH region that was sequenced in Chapter 7 was amplified as described in Section 7.1. The volume of template was as mentioned in Section 8.1.4.1. The *C.vomitoria* larvae killed by submersion and then kept in moist conditions were diluted before amplification.

8.1.5 Agarose Gel

PCR products (10µl) were separated by electrophoresis on a 1% agarose gel and visualised under UV light after staining with ethidium bromide.

8.1.6 Sequencing

The third stage larvae samples for both species were also sequenced as in Section 6.1.4. These were chosen for further analysis, to ensure the COI amplicons evident in the agarose gel pictures matched the partial COI gene sequences established in Section 6.2.1. These samples in particular were chosen after Benecke (1998) who noted that only post feeding larvae should be used in analysis to ensure the crop (gut) of the larvae were naturally empty of food, which may cause external DNA contamination. The chromatograms produced after sequencing would indicate any contamination.

8.2 Results

The states of burnt and submerged larvae before DNA extraction are presented in Figure 8.2.

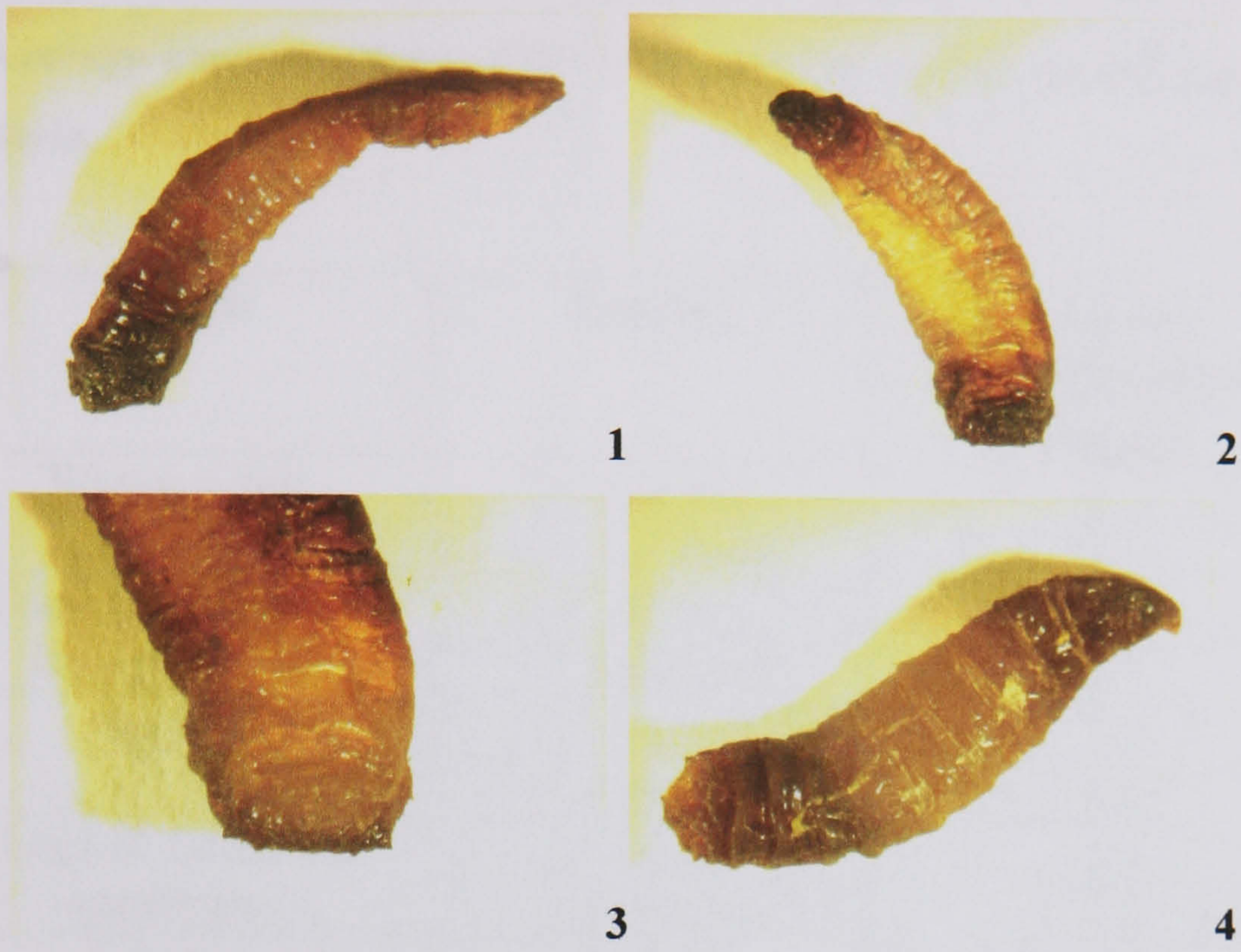


Figure 8.2a. Condition of *C. vomitoria* larvae after burning. Pictures 1, 2 and 3 show larvae that dried out and shrivelled after cooling . Picture 4 illustrates a larva that retained similar dimensions after burning.

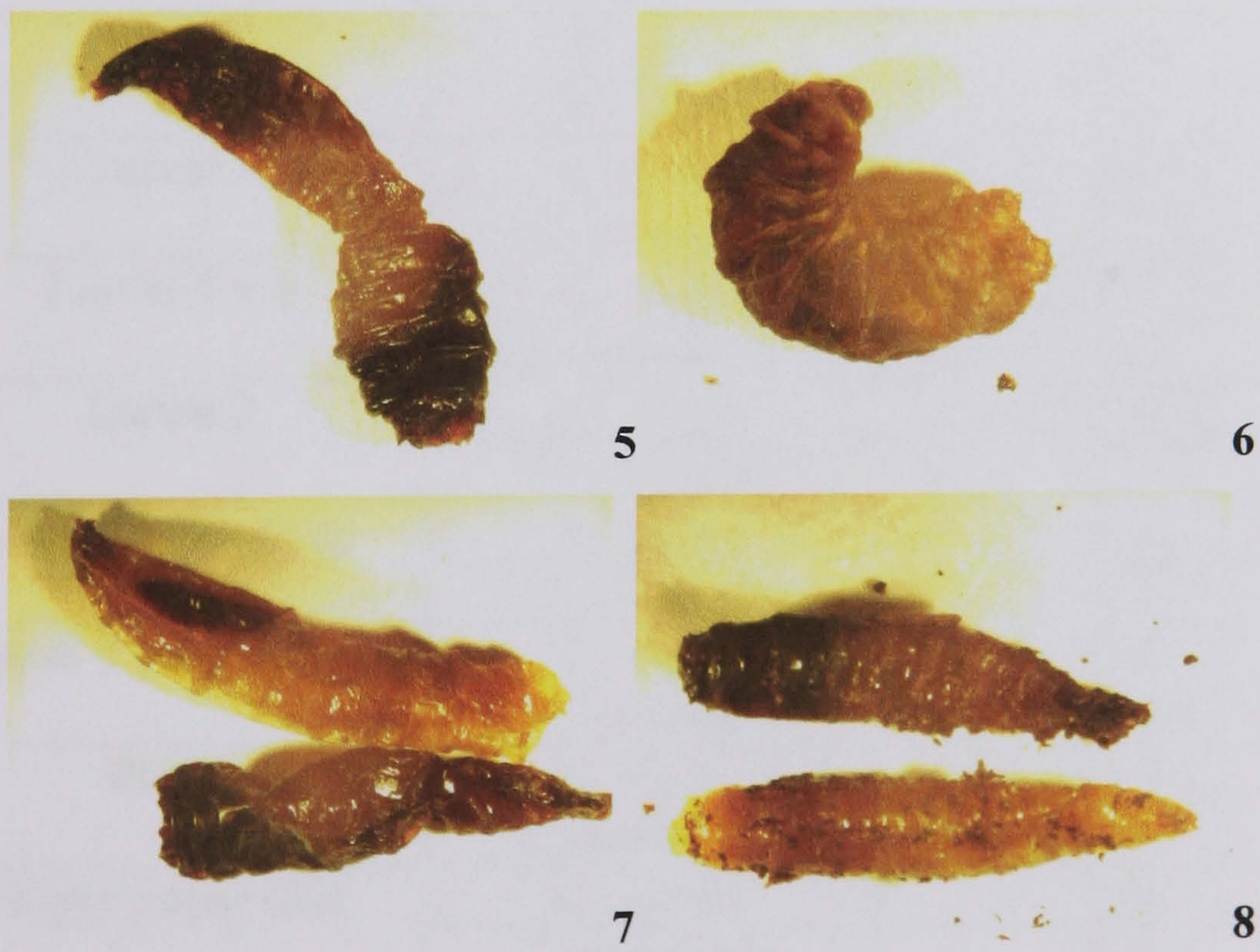


Figure 8.2b. Condition of *C. vomitoria* larvae after submersion and decomposition. Pictures 5, 6 and 7 show larvae that dried out compared to Picture 8 where the larvae were kept in moist conditions.

8.2.1 Quantification of DNA Extracts

Raw data for the PicoGreen quantification are in Appendix VI. Average concentrations per sample type for both species are presented in Table 8.2.

Table 8.2. Average concentrations of DNA extracts for various sample types of *C. vicina* and *C. vomitoria*.

Sample	Species	Average Concentration (ng/μl)
Whole Adult	*C. vicina*	12.3
	C. vomitoria	18.2
Thorax (wings and legs removed)	*C. vicina*	12.8
	C. vomitoria	16.7
Leg (1)	*C. vicina*	2.8
	C. vomitoria	1.4
Legs (6 from one specimen)	*C. vicina*	5.0
	C. vomitoria	8.5
Leg Part	*C. vicina*	2.3
	C. vomitoria	1.1
Wing	*C. vicina*	<0.3
	C. vomitoria	<0.3
Egg	*C. vicina*	1. 4
	C. vomitoria	5.1
8 Eggs	*C. vicina*	6.4
	C. vomitoria	7.3
Larval 1	*C. vicina*	2.9
	C. vomitoria	1.7
Larval 1 x 6	*C. vicina*	6.9
	C. vomitoria	7.7
Larval 2	*C. vicina*	5.9
	C. vomitoria	5.7
Larval 3 (feeding)	*C. vicina*	9.4
	C. vomitoria	9.3
Larval 3 (wandering)	*C. vicina*	10.5
	C. vomitoria	11.0
Pupal	*C. vicina*	11.2
	C. vomitoria	11.4
Empty pupal case	*C. vicina*	0.6
	C. vomitoria	1.4
Burnt Prepupal Larvae	*C. vomitoria*	13.2
Decomposing Prepupal Larvae	*C. vomitoria*	14.0

8.2.2 PCR Amplification

8.2.2.1 COI

Amplification using COI primers as shown in Table 6.1 produced DNA products of about 500 basepairs (Figure 8.3). All the different life stages for both species produced amplicons for the partial COI region. The only samples that did not produce amplicons initially were the submerged larvae that were left in moist conditions (Figure 8.3g).

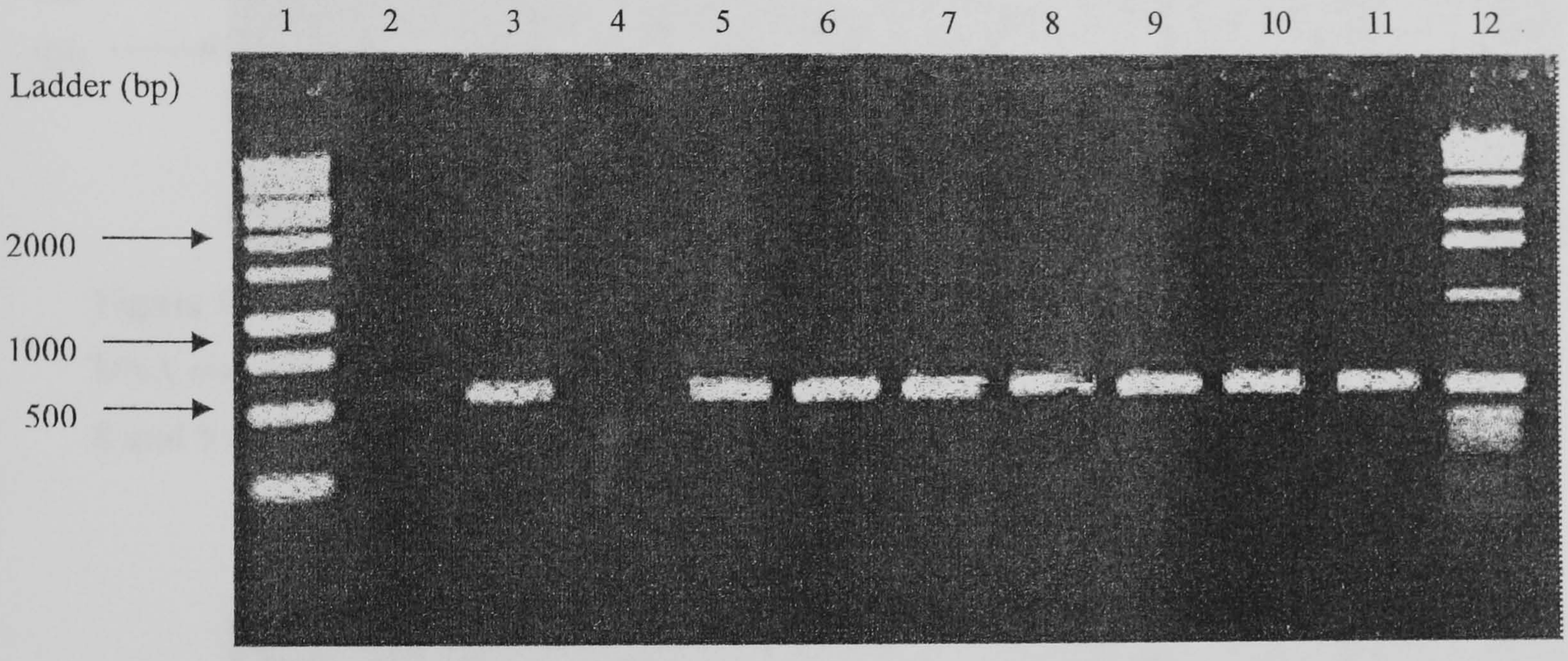


Figure 8.3a. *Calliphora vicina* amplicons of partial COI region. Lanes 1 and 12 contain DNA size markers. Lane 2 is Egg; Lane 3 is 8 Eggs; Lane 4 is Larval 1; Lane 5 is Larval 1 x 6; Lanes 6 and 7 are Larval 2; Lanes 8 and 9 are Larval 3; Lanes 10 and 11 are Prepupae.

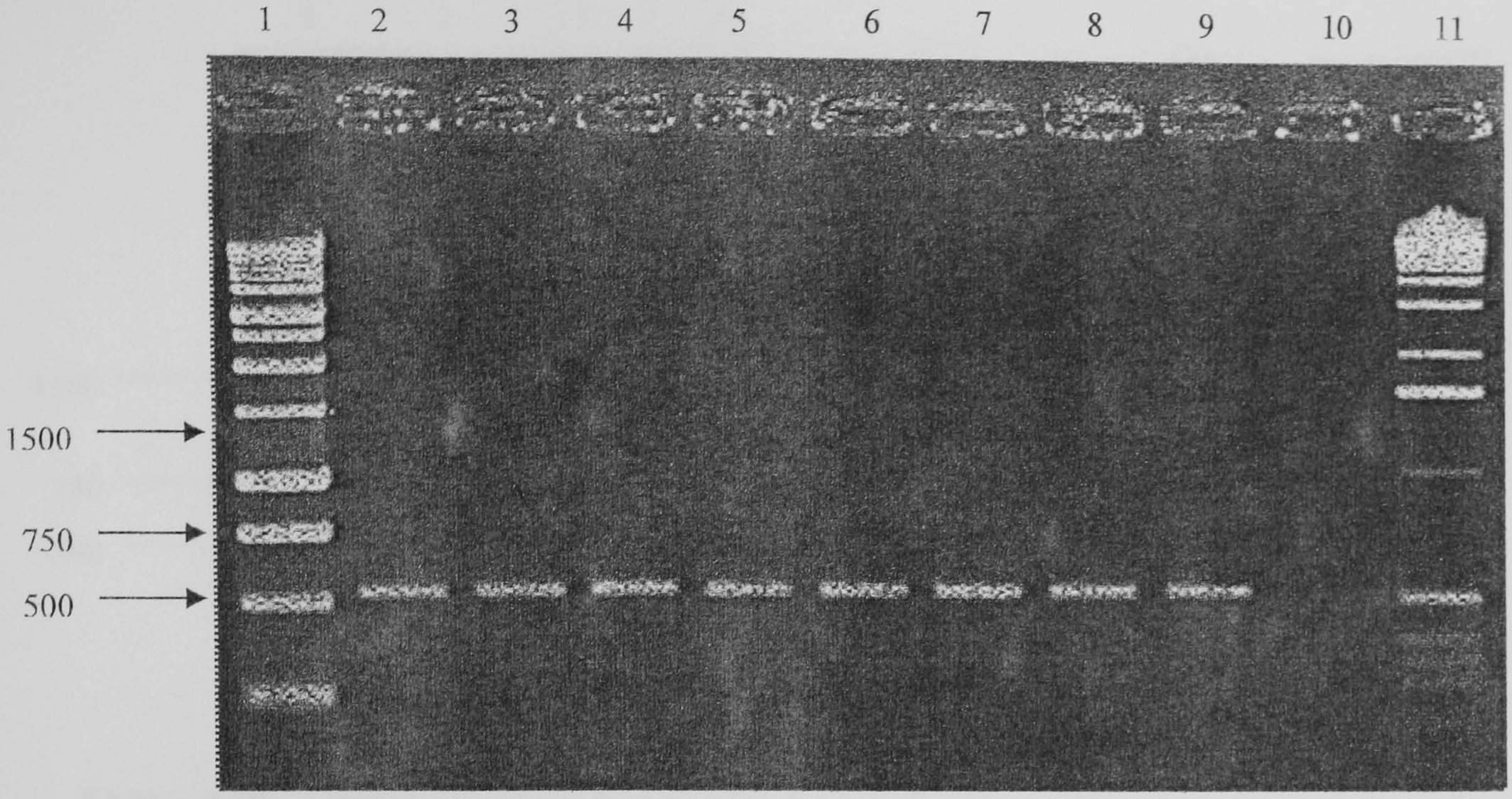


Figure 8.3b. ***Calliphora vicina*** **amplicons of partial COI region. Lanes 1 and 11 contain DNA size markers. Lanes 2 and 3 are Pupae; Lanes 4, 5 and 6 are Pupal cases; Lanes 7, 8 and 9 are adults; Lane 10 is a negative control.**

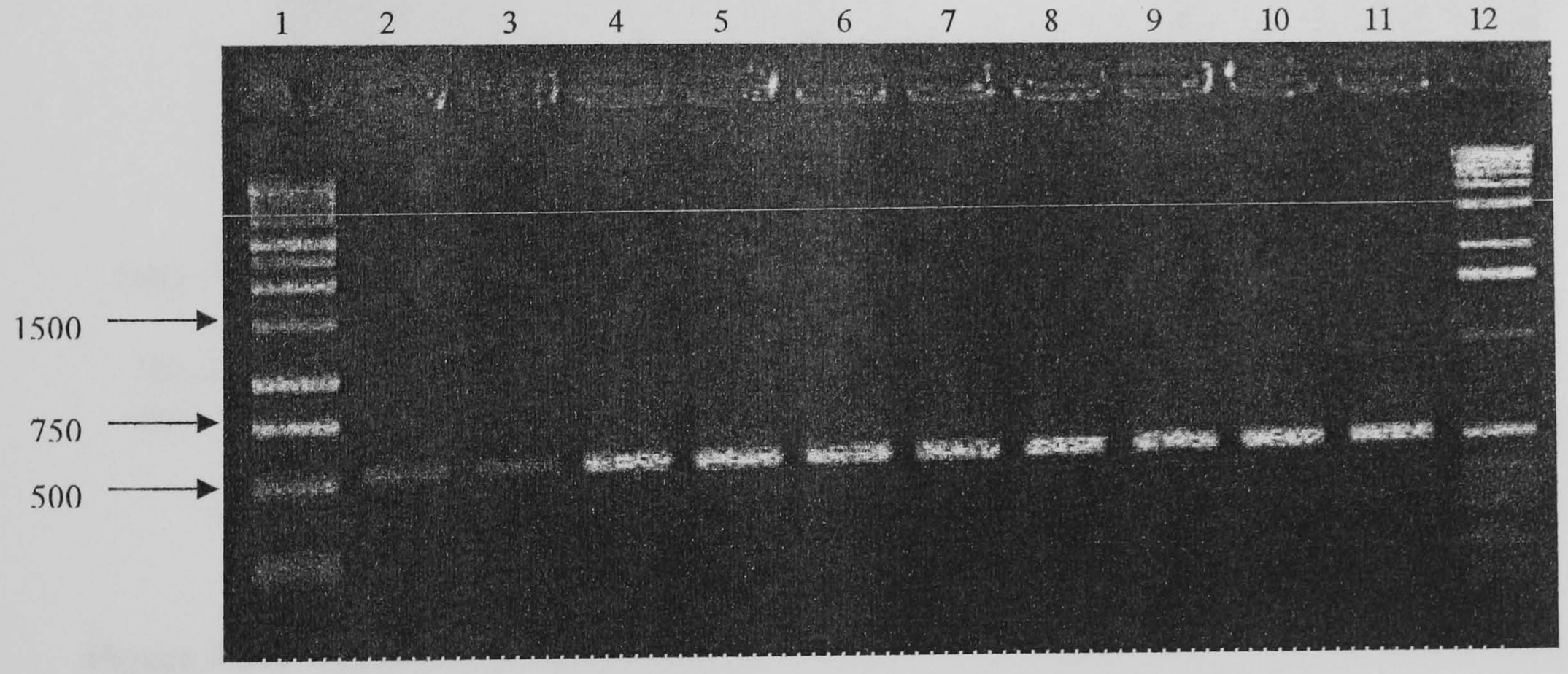


Figure 8.3c. ***Calliphora vomitoria*** **amplicons of partial COI region. Lanes 1 and 12 contain DNA size markers. Lane 2 is Egg; Lane 3 is 8 Eggs; Lane 4 is Larval 1; Lane 5 is Larval 1 x 6; Lanes 6 and 7 are Larval 2; Lanes 8 and 9 are Larval 3; Lanes 10 and 11 are Prepupae.**

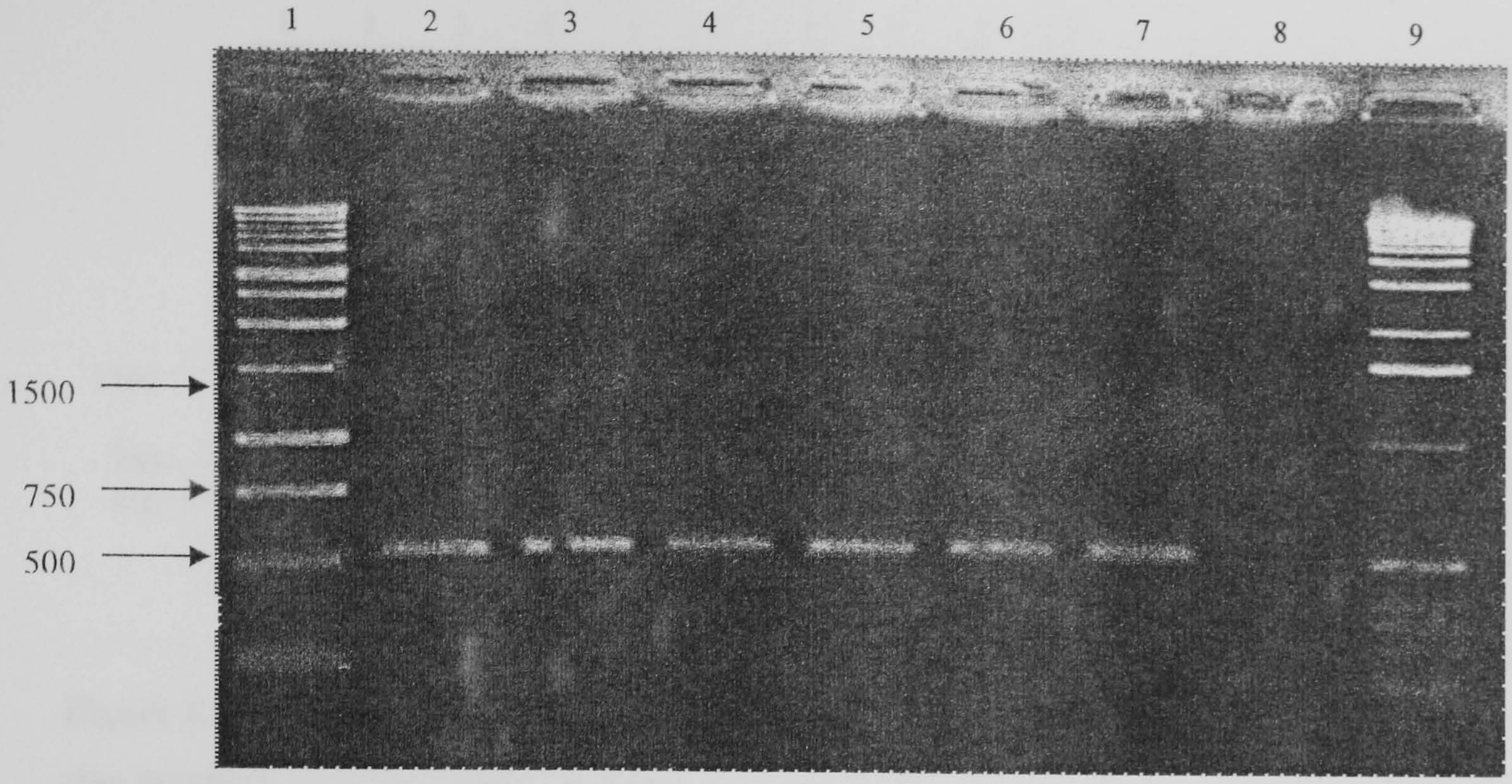


Figure 8.3d. ***Calliphora vicina*** **amplicons of partial COI region. Lanes 1 and 9 contain DNA size markers. Lanes 2 and 3 are Pupae; Lanes 4 and 5 are Pupal cases; Lanes 6 and 7 are adults; Lane 8 is a negative control.**

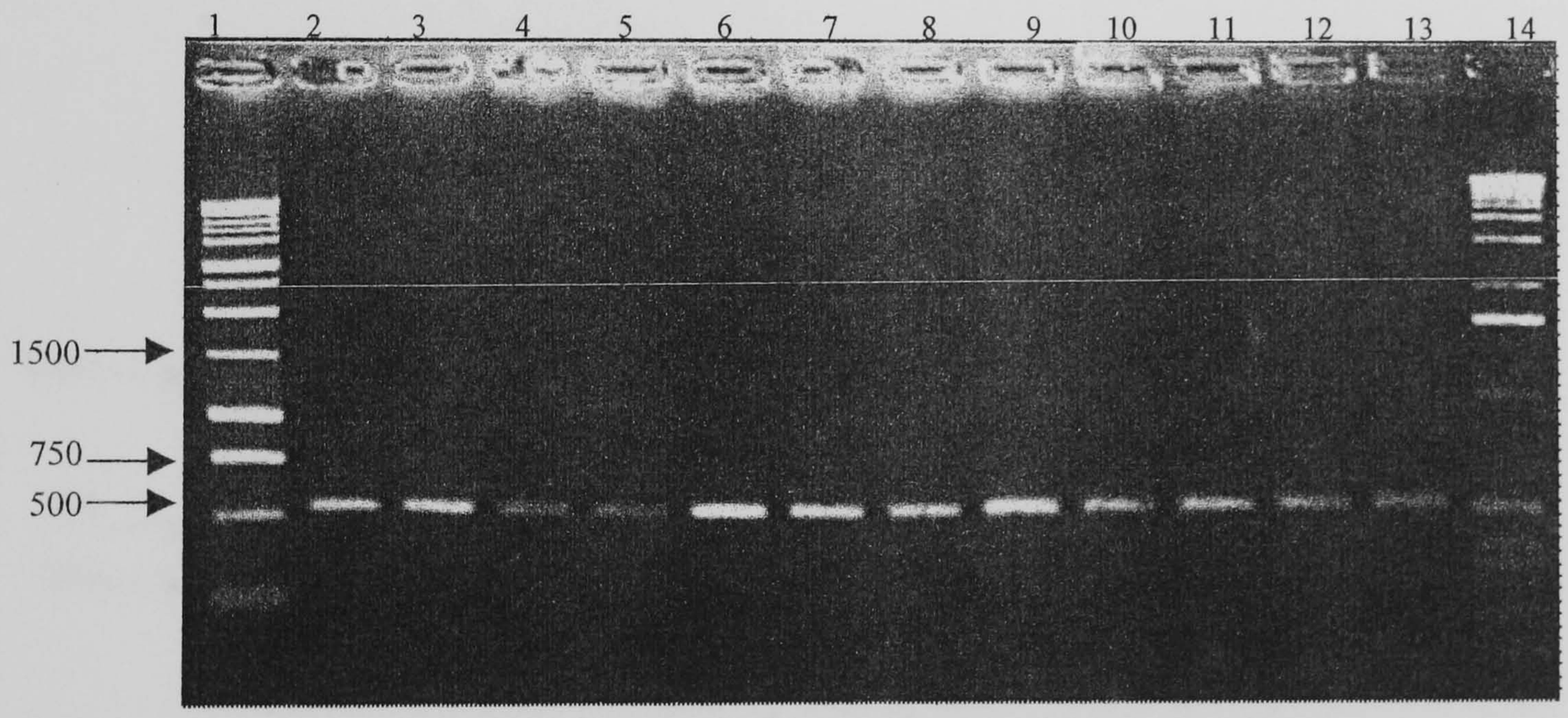


Figure 8.3e. ***Calliphora vicina*** **partial COI amplicons. Lanes 1 and 14 contain DNA size markers. Lanes 2 and 3 are part Leg amplicons; Lanes 4 and 5 are 1 Leg; Lanes 6 and 7 are 6 Legs; Lanes 8 and 9 are Wings; Lanes 10 and 11 are Thorax; Lanes 12 and 13 are whole fly.**

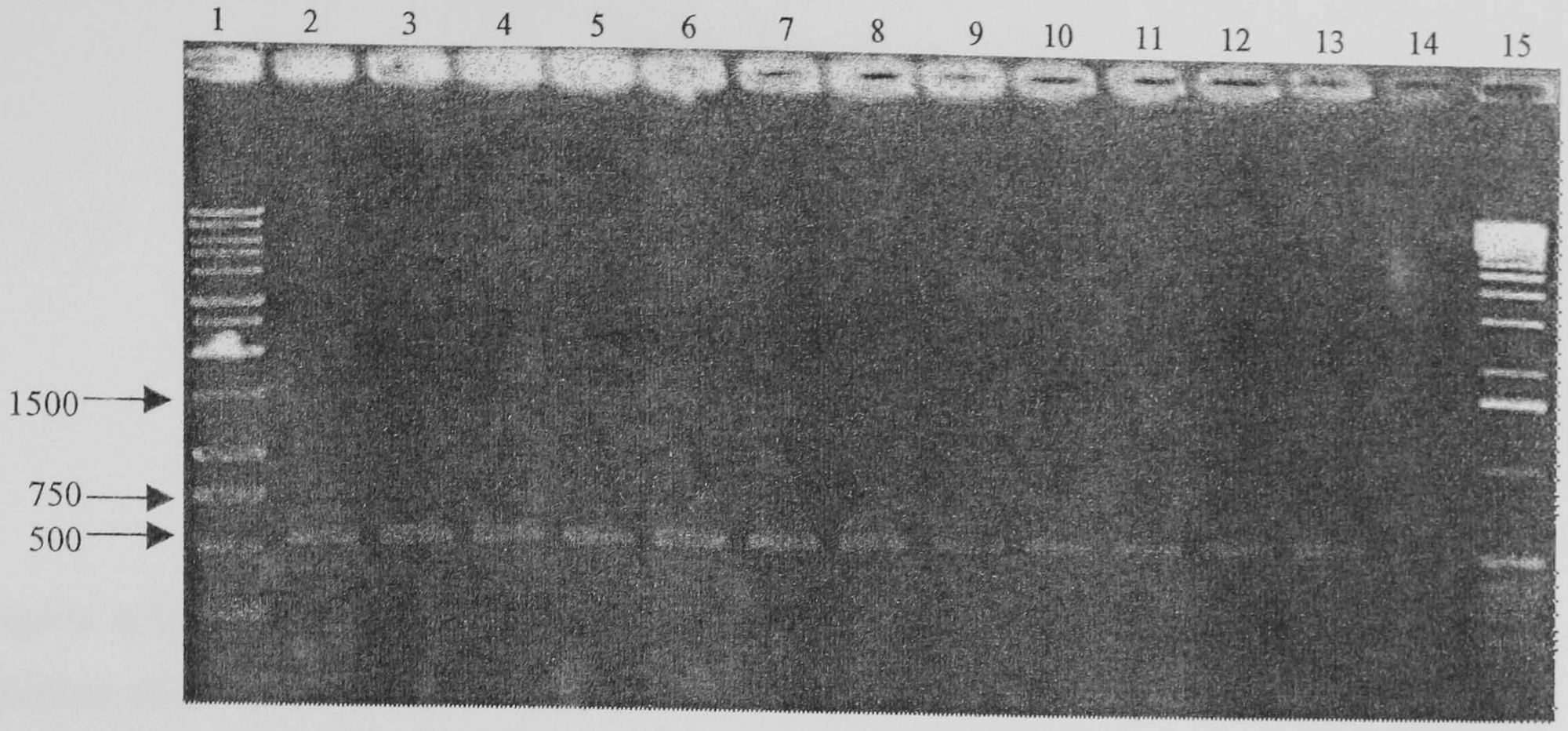


Figure 8.3f. ***Calliphora vomitoria*** **partial COI amplicons. Lanes 1 and 15 contain DNA size markers. Lanes 2 and 3 are part Leg amplicons; Lanes 4 and 5 are 1 Leg; Lanes 6 and 7 are 6 Legs; Lanes 8 and 9 are Wings; Lanes 10 and 11 are Thorax; Lanes 12 and 13 are whole fly; Lane 14 is a negative control.**

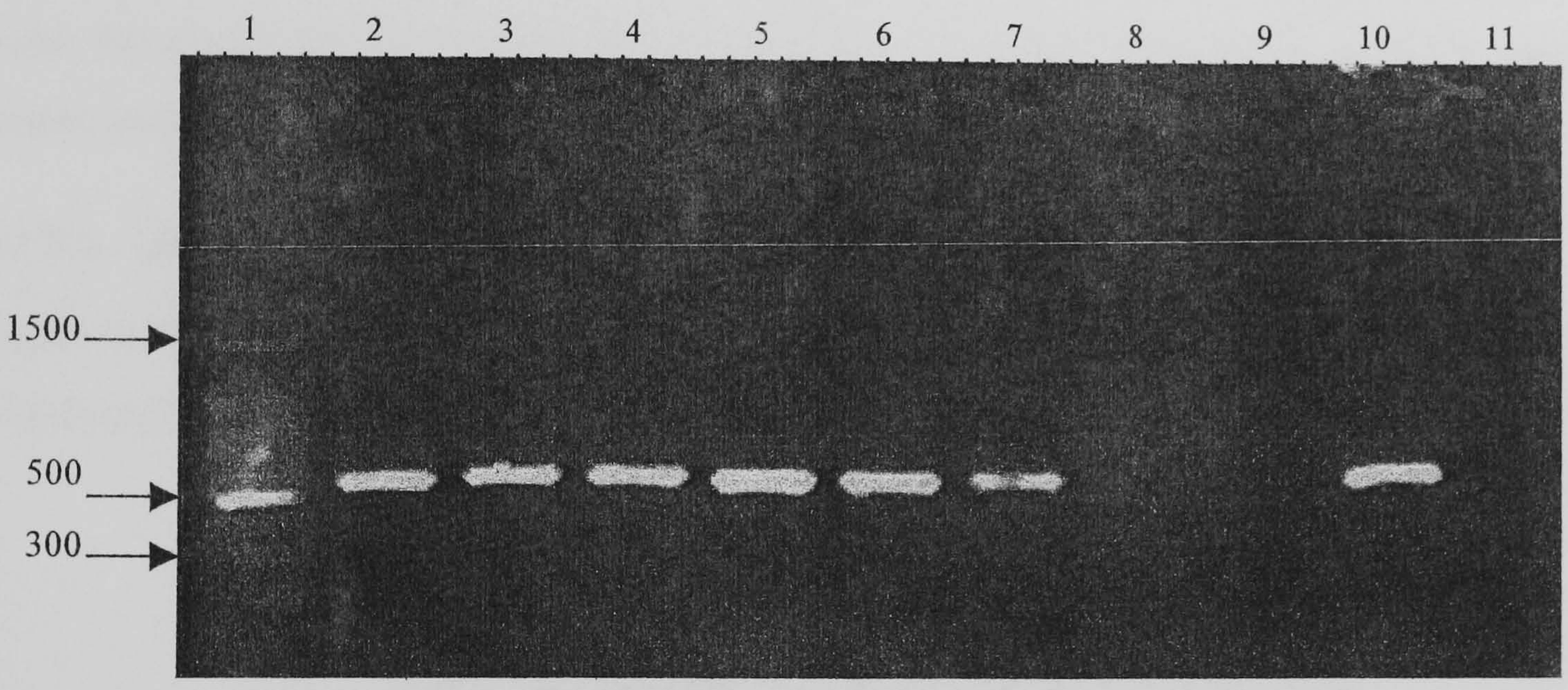


Figure 8.3g. Partial COI amplicons. Lane 1 is a DNA size marker. Lanes 2, 3 and 4 are the burnt larvae. Lanes 5, 6 and 7 are the submerged larvae that were dried out post-mortem. Lanes 8, 9 and 10 are submerged larvae that were kept moist. Lane 11 is the negative control.

The COI PCR was repeated for the submerged (moist) samples. Template volumes were varied from 3µl to 5,10 and 3µl (1 in 5 dilution). The 5 and 10µl yielded negative results (data not presented) but the 1 in 5 dilution is shown in Figure 8.3h.

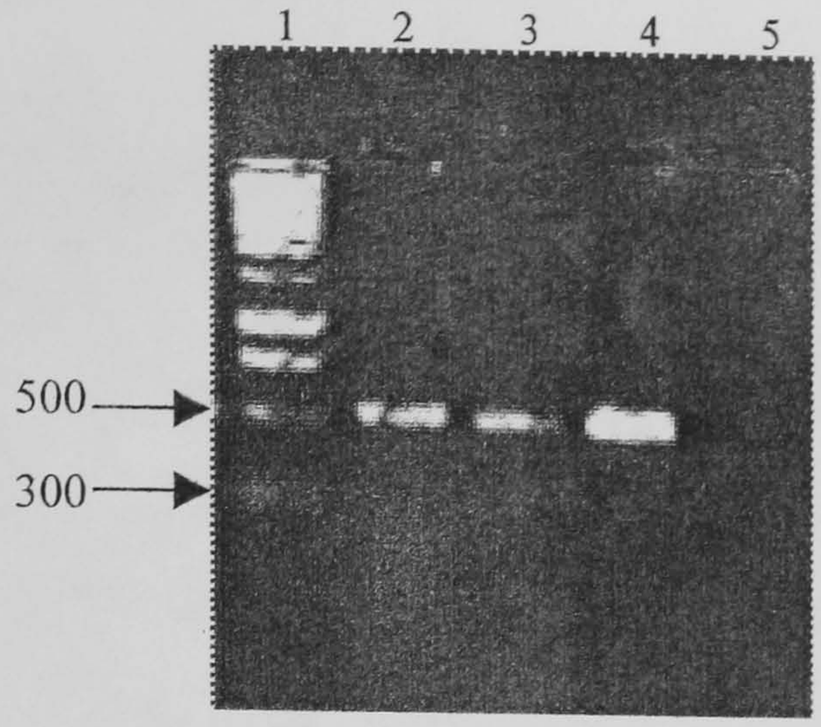


Figure 8.3h. Submerged (moist) larval samples COI amplicons using 3µl of 1 in 5 dilution of DNA template. Lane 1 is DNA ladder. Lanes 2, 3 and 4 amplicons. Lane 5 negative PCR control.

The third stage of the larval instar (feeding) partial COI amplicons were sequenced (data not shown) and all sequences had 100% identity to the respective species consensus sequences established in Chapter 6. The chromatograms were also assessed by eye to ensure no contamination was present (that would be evident by pull-up under the peaks well above the baseline noise). All chromatograms were of the same nature as those seen in the other COI work.

8.2.2.2 XDH

Figure 8.4 shows that the nuclear gene region XDH can be amplified in the samples with low DNA concentrations (Table 8.2).

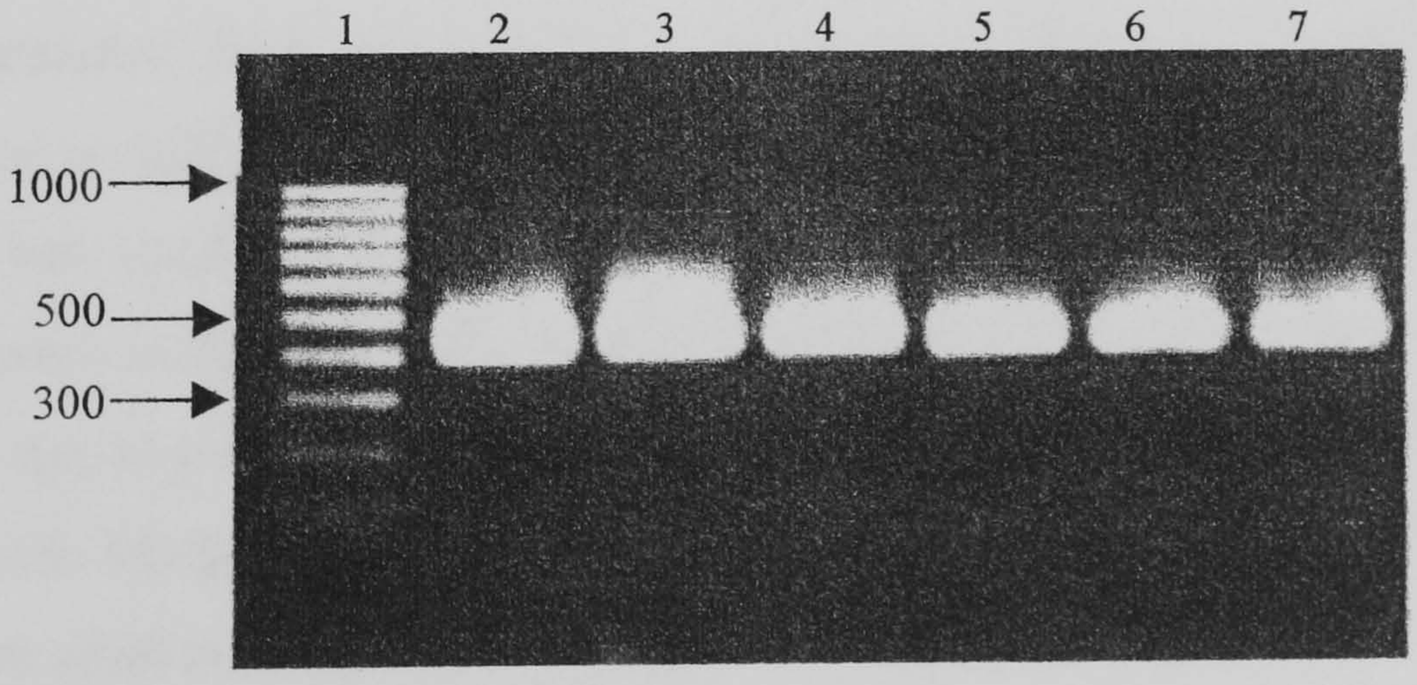


Figure 8.4a. *Calliphora vicina* and *C. vomitoria* amplicons of partial XDH region. Lane 1 contains 100bp DNA size marker. Lane 2 is *C. vicina* part of leg; Lane 3 is *C. vomitoria* part of leg; Lane 4 is *C. vicina* wing; Lane 5 is *C. vomitoria* wing; Lane 6 is *C. vicina* pupal case and Lane 7 is *C. vomitoria* pupal case.

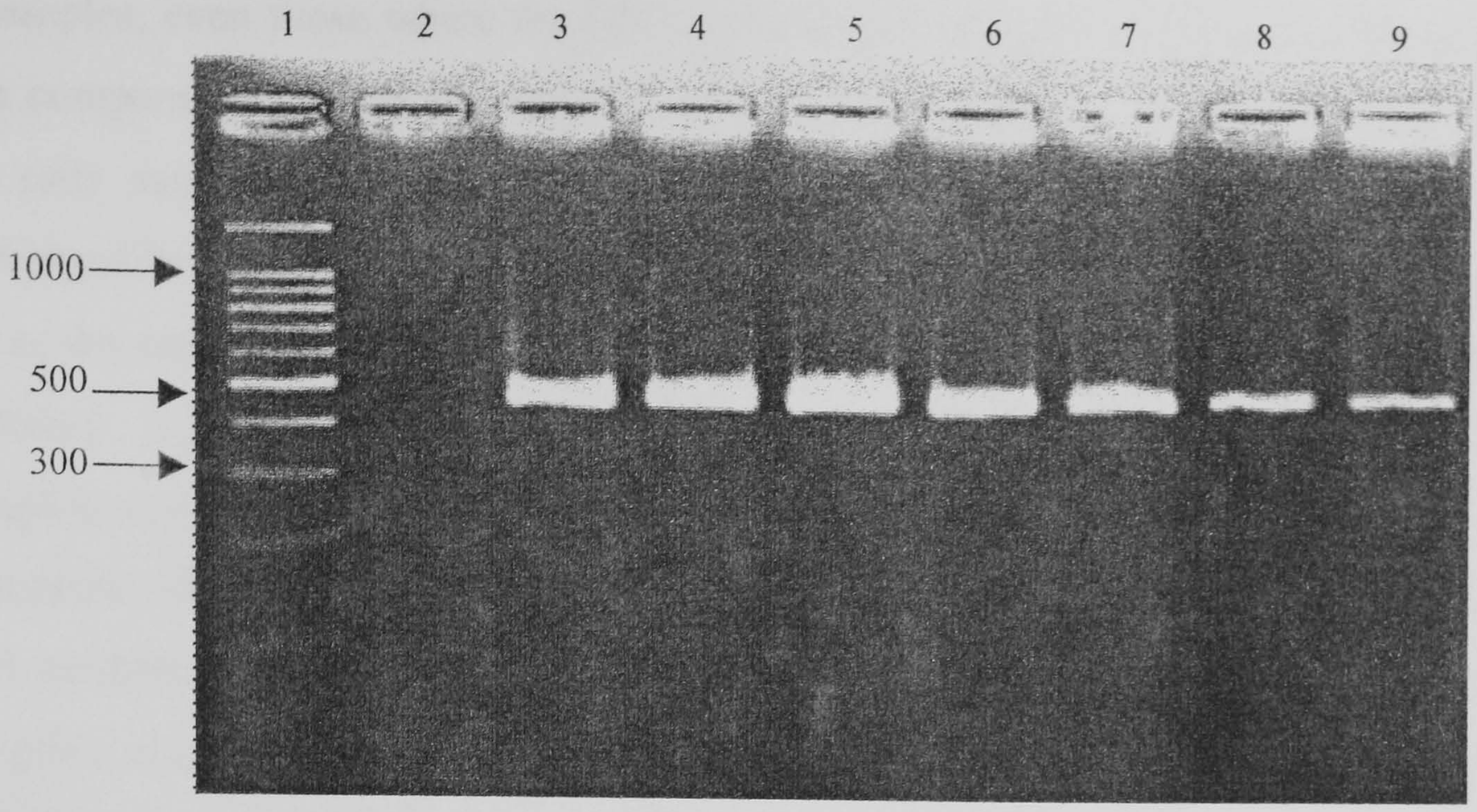


Figure 8.4b. ***Calliphora vomitoria*** **amplicons of partial XDH region. Lane 1 contains 100bp DNA size markers. Lane 2 is negative control; Lane 3 is positive control (adult thorax); Lanes 4 and 5 are larvae killed by submersion and then kept in moist conditions; Lanes 6 and 7 are submerged and then dried larvae; Lanes 8 and 9 are larvae killed by burning.**

8.3 Discussion

The purpose of this part of the study was to ensure that the molecular markers established in the previous Chapters would apply to other types of insect sample and not just the adult and wandering third instar as used when ascertaining the markers. This included larvae that had been burnt and those that were decomposing. The photographs indicate the damage caused to the external appearance of the larvae.

DNA was extracted from all samples in sufficient amounts to be quantified using PicoGreen and amplified using the COI primers. This was true for the empty pupal cases, where low levels of DNA were expected. The wing, another body part where DNA levels were likely to be low, yielded insufficient DNA for accurate quantitation but still gave rise to amplicons as visualised by agarose gel electrophoresis. This is in accordance with Malgorn and Coquoz (1999) who demonstrated that DNA could be extracted from adult structures.

Quantification does not give any indication of the quality of the DNA, therefore a mitochondrial and nuclear gene amplification was carried out to assess whether fragments could be amplified from these samples. The fragment could be amplified in

all samples, even those where the DNA concentrations had been very low (these had been compensated for by the addition of more DNA template in the PCR reaction). The only samples that did not work particularly well at first attempt were the submerged larvae that had remained in moist conditions after death and thus were not able to dry out. Under these conditions bacterial endonucleases can degrade the DNA rendering it too fragmented for successful amplification. However, after reamplification using a dilution of the DNA as a template, amplicons of the appropriate size were evident. It appears that the PCR reactions were overloaded with DNA template initially. If too much DNA is added to a PCR reaction, all the primers and other constituents of the reaction mix can get exhausted in the early cycles of PCR, thus not amplifying the COI fragment. Alternatively, if a PCR inhibitor was present in the DNA samples, the dilution of the sample may have diluted this factor enough so that inhibition no longer occurred.

Malgorn and Coquoz (1999) note that mitochondrial DNA markers should be used when dealing with small parts of insects. This work has shown that whilst mtDNA consistently amplifies in all samples, it is still possible to amplify nuDNA regions in samples that have low extracted DNA quantities.

It would be interesting to examine whether fragments longer than 530bp could be amplified from these samples (especially the necrosing larvae where DNA degradation is likely to be greater). Schroeder *et al.* (2003a) discovered on amplification of a COI and COII fragment (2.4kb) in *C. vicina*, *L. sericata* and *C.vomitoria* samples, that they could only amplify those samples that had been frozen alive; there was enough DNA degradation in dead flies to prevent amplification of a fragment of this length. Wells and Sperling (2001) amplified the equivalent 2.3kb fragment and they too could not amplify this sequence in an 18-year-old dry specimen. They could only obtain a complete 304bp fragment. It does appear that there is a finite length of DNA fragment that can be amplified even for the prevalent mtDNA genome.

Vincent *et al.* (2000) obtained DNA, after phenol chloroform extraction, that was of good enough quality to amplify a 272 and 229 base pair fragment (*C. vicina* and *C.vomitoria* respectively) from empty puparia, eggs and poorly preserved flies. The

researchers postulated that it therefore might be possible to obtain DNA from legs and wings. This has been realised in this work.

The third larval stage (feeding) samples that were sequenced produced no anomalies when compared to the consensus sequences. They also indicated no evidence of DNA contamination, which has been previously suggested, might hinder DNA molecular identification. This could of course be due to the specificity of the COI primers used in amplification.

From this work it appears therefore that the COI and XDH molecular markers established are viable for forensic useas it has also been shown that they can be used on a variety of adult body parts and developmental stages.

Chapter 9

Examination of the *Calliphora vicina* and *Calliphora vomitoria* Mitochondrial Control Region

The mitochondrial genome contains relatively little non-coding DNA when compared with the nuclear genome. Aside from intergenic spacers, which consist of a few nucleotides, the majority of non-coding mtDNA is located in the control region. This region is believed to be involved in the regulation in the transcription and control of mtDNA replication (Clayton 1982). In vertebrates this region is also known as the displacement loop (D-loop). The human D-loop was sequenced by Anderson *et al.* (1981). There are two variable regions in this sequence, known as hypervariable region (HV-I and HV-II). These regions are used for identification purposes in forensic science. This method of identification is used when traditional nuclear DNA profiling is not possible e.g. in degraded samples. It has also used as a population level marker in vertebrates (Simon *et al.* 1994).

The control region in invertebrates is known as the A+T rich region due to the high level of adenine and thymine residues (85-96%, Zhang and Hewitt 1997b). The A+T rich region has shown variability within Insecta. This variability exists on several levels. Firstly, the relative location of the control region compared to the protein coding genes and tRNA coding regions vary within invertebrate mtDNA. Even within Insecta the flanking genes can be different, e.g. in Diptera it is flanked by $tRNA^{ile}$ and small rRNA (also known as 12S RNA or srRNA; Figure 3.1), in Hymenoptera $tRNA^{glu}$ and srRNA and in Lepidoptera $tRNA^{met}$ and srRNA (Zhang and Hewitt 1997b). Secondly, the control region also shows differences in the location of regions of sequence conservation and variation. There are regions of conservation between species. Zhang and Hewitt referred to these as structural elements and these regions have been identified in a variety of insects, which probably reflects the functional importance of these regions. Five structural elements were identified through comparative analysis of dipteran and orthopteran control regions (Zhang *et al.* 1995). The pattern of sequence conservation varies among species. Zhang and Hewitt (1997b) split Insecta into two groups according to the layout of the structural regions.

In Group 1 (Diptera - *Drosophila* species) the control region comprises of two domains: a conserved sequence block near the $tRNA^{ile}$ gene alongside a more variable domain at the 5' end (Clary & Wolstenholme 1985, Zhang and Hewitt 1997b). In contrast, in Group 2 (Orthoptera, Diptera – *Anopheles* species and Lepidoptera), conserved sequence blocks are dispersed throughout the entire control region (Taylor *et al.* 1993, Zhang *et al.* 1995, Zhang and Hewitt 1997b).

Lessinger and Azeredo-Espin (2000) also reported on two distinct domains, after aligning the control regions from *C. hominivorax*, *C. macellaria* and *C.megacephala*. Domain 'A', which is equivalent to the conserved sequence block near the $tRNA^{ile}$ coding region, contains eight conserved sequence blocks between species. Domain 'B' was identified at the 5' end of the control region. This region is more variable in length, ranging from 393 to 794bp for the species examined. This region does not have conserved regions.

The A+T rich region can vary greatly in length between species. This has often been shown to be due to the presence of tandem repeat sequences. These can differ in size and number within the region. Total length variation can be heteroplasmic i.e. it may vary within an individual. Heteroplasmy has been shown to be at very high levels in some individuals. Rand and Harrison (1989) showed that 35% of the total mtDNA diversity in crickets was within individuals and greater than 50% within populations. They reported that the amount of mitochondrial length variation between populations was very low. Length of the control region may therefore not be a useful feature for differentiating between populations and sequence variation is more likely to provide more useful information.

The high A and T base content and extensive size variation in the insect control region make sequencing this region difficult (Zhang and Hewitt 1997b), e.g. *D. melanogaster* has a 4601bp A+T rich region compared to 520bp in *Anopheles gambiae* Giles (figures taken from Zhang and Hewitt 1997b). Yamauchi *et al.* (2004) were unable to sequence the control region of the cockroach (*Periplaneta fuliginosa*) as the secondary structure and high A and T nucleotide levels made sequencing difficult. Sequencing however, is much more efficient than RFLP for the control region as the high level of A and T reduces the number of restriction enzymes available. Most enzymes have a

cut site that includes at least one G and C nucleotide.

This region has been characterised in only a few insect groups despite its potential usefulness for population level studies. Schultheis *et al.* (2002) have shown that the mitochondrial control region was useful for determining genetic differentiation among cohorts and distinguishing geographically isolated populations of *Peltoperla tarteri* Stark and Kondratieffe.

The control region for *Calliphora* species has not been sequenced and this was the initial aim of this section of work. It was then intended to compare the control regions of *C. vicina* and *C. vomitoria* from different geographic locations.

9.1 Materials and Methods

9.1.1 DNA Sample Preparation

9.1.1.1 Total DNA extractions

DNA was extracted as described in Section 5.2. For the initial amplifications of the complete A+T rich regions and for the nested PCR *C. vicina* from Waterloo and *C.vomitoria* from a commercial supplier were used. In the later amplifications of the conserved and variable regions samples from different *C. vicina* and *C. vomitoria* populations were included. Only adult flies were used in the control region experiments.

9.1.1.2 Separate nuclear and mitochondrial DNA extractions

Whilst the majority of extractions in this work were conducted as in Section 5.2, three *C. vicina* adult thoraxes were homogenised in 450µl TEN buffer and 40µl of 20% w/v SDS. Samples were then centrifuged at 100 x g for 1 min to spin down cell debris, the supernatants were transferred to fresh tubes and the samples were centrifuged at 600 x g for 10 min to pellet the nuclei. The supernatants were transferred to fresh tubes and these tubes were spun at 15,000 x g for 5 min to pellet the mitochondria (centrifugation speeds and durations after Lodish *et al.* 1995). The supernatants were removed and discarded. To all the tubes (containing either nuclei or mitochondrial pellets) buffer and Proteinase K was added as per Section 5.2.3. Extraction continued

as described for the spin column technique in Section 5.2.3. DNA was eluted off the spin columns with 50µl ddH_2O.

9.1.2 Initial PCR of the complete A+T rich region

Primers for the amplification of this region were those utilised by Roehrdanz (1995). The primers are called TM-N-193 and SR-J-14612 (Table 9.1). The primer binding sites are located in the $tRNA^{met}$ and srRNA regions respectively (Figure 9.1).

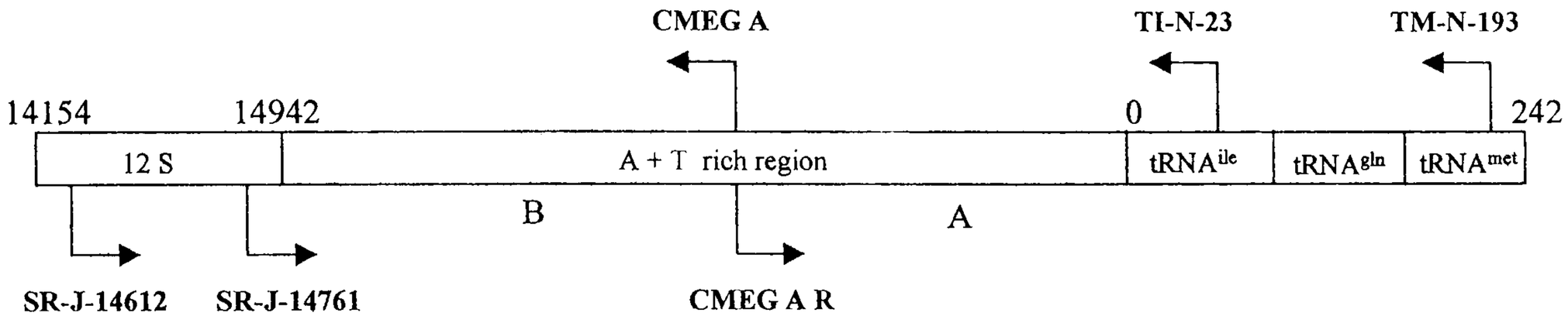


Figure 9.1. Location of primer binding sites utilised in this study in relation to the A + T rich region and surrounding genes. Arrows indicate direction of DNA amplification, hence those above the gene diagram are reverse primers and those below the diagram are forward primers. Numbers indicate position relative to the D. yakuba sequence (Clary and Wolstenholme 1985).

Table 9.1. Sequence of primers to amplify the mitochondrial control region.

Primer Name	Sequence of Primer
SR-J-14612	5' AGGGTATCTAATCCTAGTTT 3'
TM-N-193	5' TGGGGTATGAACCCAGTAGCTT 3'

Previous studies on A and T rich sequences have discovered that a reduced elongation temperature is needed to provide reliable amplification of this region (Su *et al.* 1996). A gradient PCR was conducted, as in Section 5.4, to find the optimum annealing temperature for this primer pair (Table 9.1). The following thermal regime was carried out on a Peltier Tetrad Thermal Cycler (MJ Research) 94°C for 2 min; [94°C for 30s, 42-60°C for 1 min, 60°C for 2 min] for 35 cycles; 60°C for 10 min.

The PCR products were run on a 1% agarose gel, stained with ethidium bromide and viewed under UV illumination. The most stringent annealing temperature was found

to be 57.7°C. An annealing temperature of 57°C was therefore used in further amplifications.

The mitochondrial control region was amplified in ten *C. vicina* and ten *C. vomitoria* samples.

9.1.2.1 Purification

Gels were run post-amplification and purification was carried out using GFX PCR DNA and Gel Band Purification Kit spin columns as in Section 5.5. Samples were eluted using 50µl of sterile H_2O.

9.1.2.2 Sequencing

The samples were sequenced as in Section 5.6. Sequences were compared and aligned using BioEdit software.

9.1.3 PCR of Internal A+T Region

The complete control region in these two species is too long to sequence as one fragment. A nested PCR was therefore carried out using the PCR products from the complete control region amplification as template DNA.

A forward primer was designed using Primer 3 software based upon the sequences established from the 5' end of the SR-J-14612/TM-N-193 amplicons. This new primer is located within the srRNA region and has been designated SR-J-14761 after the nomenclature in Simon *et al.* (1994). The designed primer was used in combination with the reverse primer TI-N-24 from Simon *et al.* (1994) (Figure 9.1). This primer is located in the $tRNA^{ile}$ gene and was used by Lessinger and Azeredo-Espin (2000) as their internal reverse primer (Table 9.2).

Table 9.2. Primer sequences for the nested amplification of the mitochondrial control region.

Primer Name	Sequence of Primer
SR-J-14761	5' CGTATAACCGCGGCTGCT 3'
TI-N-24	5' ATTTACCCTATCAAGGTAA 3'

The PCR conditions were the same as the SR-J-14612/TM-N-193 reaction. Instead of extracted DNA, the SR-J-14612/TM-N-193 amplicons were added as template DNA for the reactions.

The amplicons were run on a 1% agarose gel and stained with ethidium bromide to be visualised under UV light.

9.1.4 PCR of Regions A and B

As described previously, Lessinger and Azeredo–Espin (2000) concluded that the control region of Calliphoridae was split into a conserved (A) and a variable (B) region. The control region was also amplified in two separate parts using internal primers as communicated by A.C. Junqueira following the standard PCR protocol reported in Section 5.4 (Table 9.3).

Table 9.3. Sequence of primers used to amplify the A+T rich region in two parts. The primers will amplify regions A and B separately (regions as designated by Lessinger and Azeredo-Espin (2000)).

Region	Primer Name	Sequence of Primer	Used in conjunction with
A	CMEG AR	5' AATCCAGTTAAGAATATCAT 3'	TM-N-193
B	CMEG A	5' ATGATATTCTTAACTGGATT 3'	SR-J-14612

Reactions were run on the GeneAmp® PCR System 9700 Thermal Cycler for the following thermal cycles ~ 94°C for 2 min; [94°C for 30s; 50°C for 60s; 60°C for 2 min] for 35 cycles; 60°C for 10 min; 4°C to finish.

Samples were run on a 1% agarose gel to view product fragment sizes. Gels were stained with ethidium bromide before viewing under UV light.

9.1.4.1 Purification and Sequencing

Samples were purified with the GFX PCR DNA and Gel Band Purification kit and sequencing reactions were conducted as discussed in Sections 5.5 and 5.6.

9.1.4.2 Sequence analysis

Sequences were analysed as in Section 5.7.

9.1.5 PCR of separate nuclear and mitochondrial DNA

Amplifications were conducted using separate nuclear and mitochondrial DNA as templates. Partial regions of actin, COI, complete A+T rich region and conserved A+T rich region A were amplified. Actin was used as an example of a nuclear gene

and is discussed further in Chapter 13. All PCR conditions were as previously discussed for each primer pair (for actin, see Chapter 13, Section 13.1.2). Amplicons were separated on 2% agarose gels.

9.2 Results

Figure 9.2 indicates that multiple banding is present after amplification with TM-N-193 and SR-J-14612 primers for both species. A dominant band is present, under 2500bp in length, and another below 750bp.

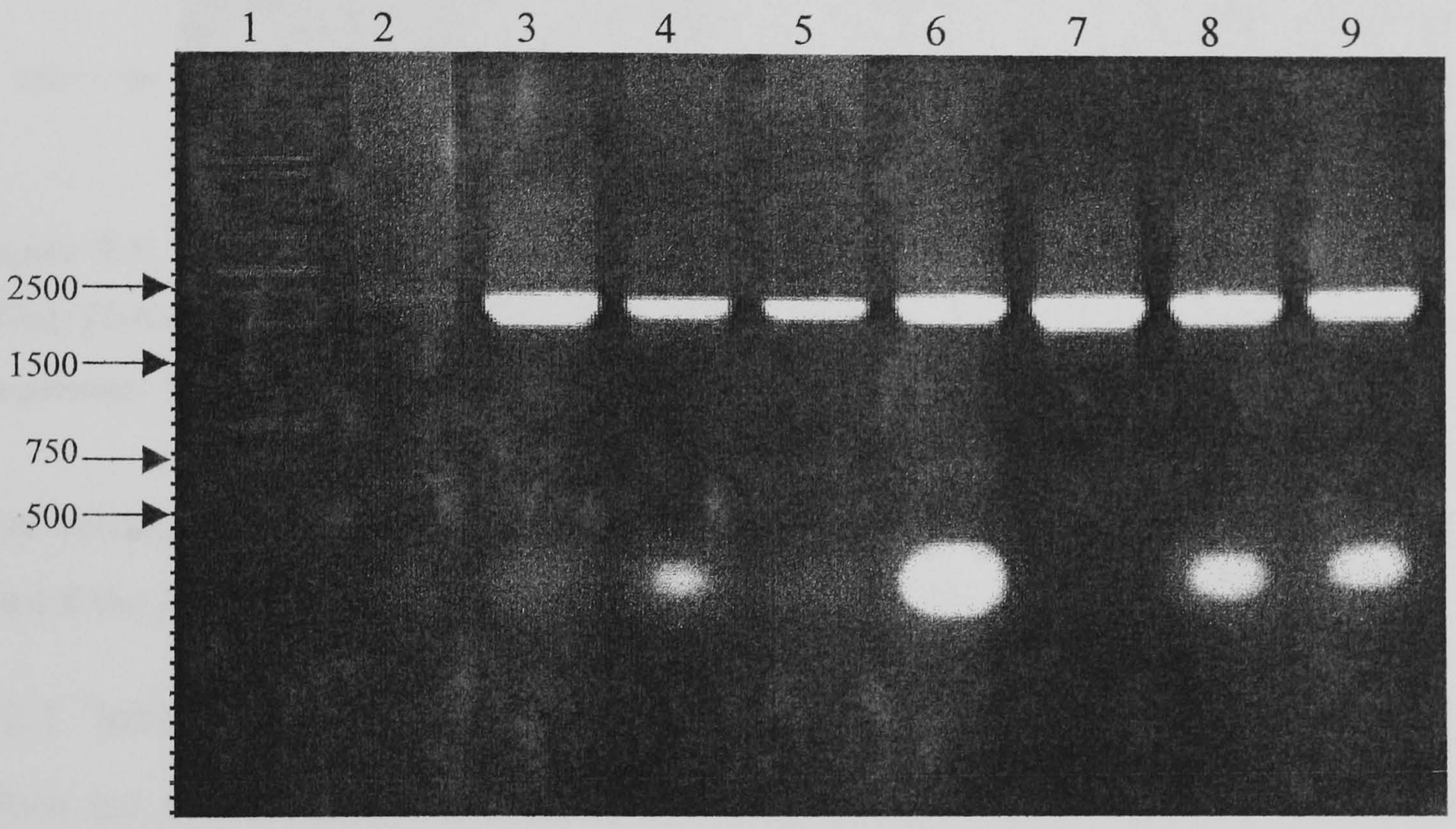


Figure 9.2. Example of amplicons produced using the primers SR-J-15612 and TM-N-193. Lane 1 contains 1KB DNA ladder. Lanes 2-5 contain *C. vicina* amplicons and Lanes 6-9 contain *C. vomitoria* amplicons.

This banding pattern was evident even when the annealing temperature was increased to improve primer binding stringency in a gradient PCR reaction.

Both bands were excised, purified and sequenced. The longer band was used in a nested PCR after a new forward primer was designed. The nested PCR also produced multiple amplicons. The largest band was around 2000bp (Figure 9.3). There is a fragment of around 1500bp and a small fragment of just over 250bp in length.

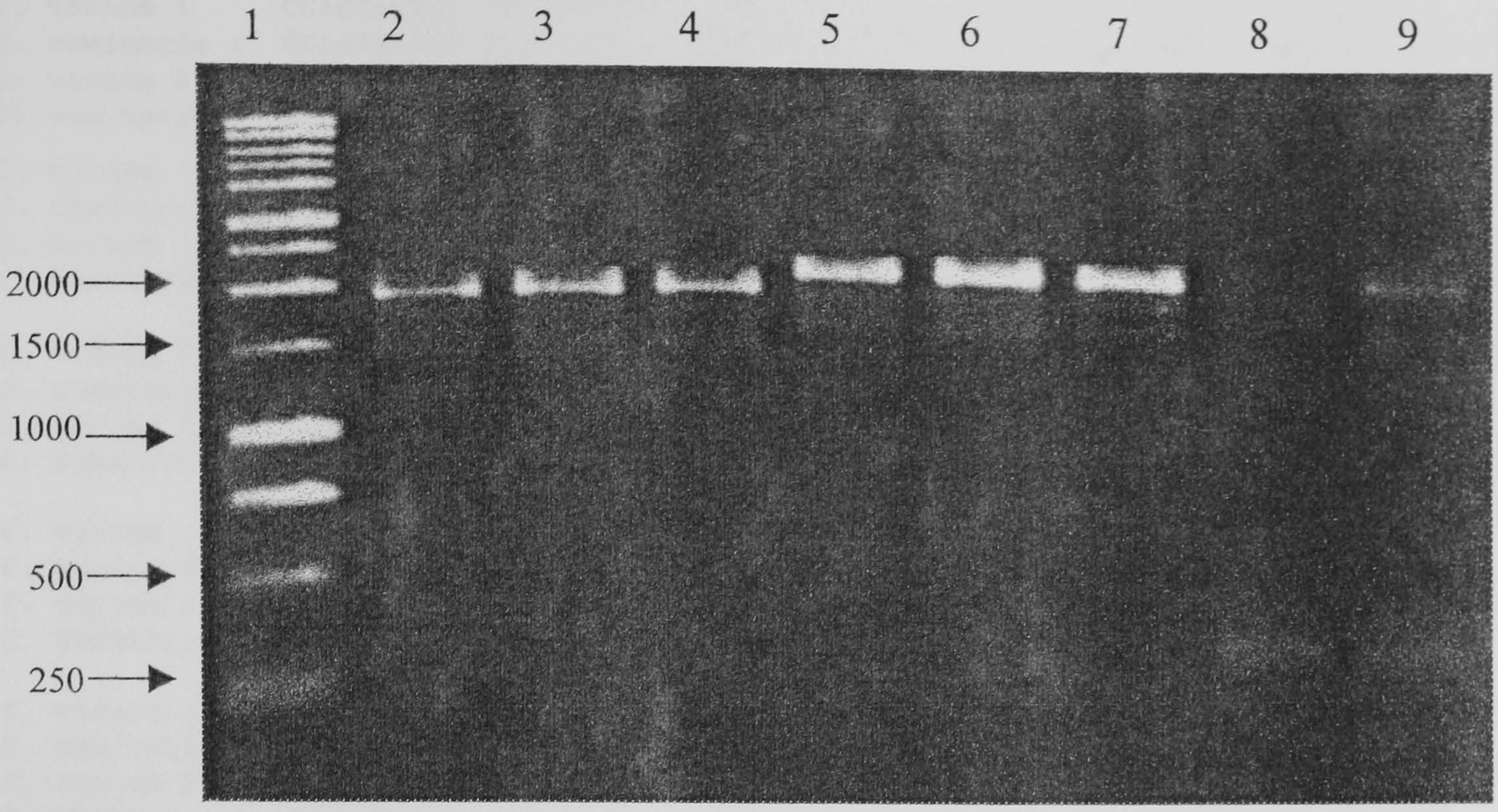


Figure 9.3. Examples of amplicons produced in a nested PCR using primers SR-J-14761/TI-N-24. Lane 1 contains 1KB DNA ladder. Lanes 2-5 contain *C. vicina* amplicons. Lanes 6-9 contain *C. vomitoria* amplicons.

Size variation between individuals within the same species also exists. In Figure 9.3 Lane 8 the dominant band at 2000bp is not present.

9.2.1 Intra-individual variation in control region

When the amplicons in the SR-J-14612/TM-N-193 and SR-J-14761/TI-N-23 were sequenced it became evident that there is some intra-individual variation in the control region. There appears to be two types of region within individuals of both species when amplified with these primers. The small amplicons (250 and 500bp) were identical (within each species) to the 5' end of the 1500bp amplicon (Figure 9.3). This sequence differed from the 5' end of the longest amplicon sequence. Figure 9.4 illustrates these differences. The long amplicon sequence has been called 'type 1' for this study and the smallest amplicon/1500 bp sequence 'type 2'

```
C. vicina 1      CCACTACTCT TTAGTATTAC TATTTCTAAG TTTCCTTAAT TAATAATATT AATTACTGCG
C. vomitoria 1   CCAATACTCT TTAGTATTAC TATTTCTAAG TTTCCTTAAT TAATAATATT AATTACTGCG
C. vicina 2      CCACTACTCT TTAGTATTAC TATTTCTAAG TTTCCTTAAT TAATAATATT AATTACTGCG
C. vomitoria 2   CCAATACTCT TTAGTATTAC TATTTCTAAG TTTCCTTAAT TAATAATATT AATTACTGCG

C. vicina 1      GATAAAATAA TTTTACTTAT TATTAAAATA AATAAAAATT CACATAAAAA TTTACATATA
C. vomitoria 1   GATAAAATAA TTTTATTTAC TATTAAAATA AATAAAAATT CACATAAAAA TTTACATATA
C. vicina 2      GATAAAATAA TTTTACTTAT TATTAAAATA AATAAAAATT CACATAAAAA TTTACATATA
C. vomitoria 2   GATAAAATAA TTTTATTTAC TATTAAAATA AATAAAAATT CACATAAAAA TTTACATATA

C. vicina 1      AATTAAACTA ACAATAAATT TACAAGCCAA AATAAAACTT TAGACAATAA ATTTAAAAAT
C. vomitoria 1   AATTAAACTA ATAATAAATT TATAAGCCAA AATAAAACTT AATACAATTA ATTTAAAATT
C. vicina 2      AATTAAACTA ACAATAAATT TACAAGCCAA AATAAAACTT TAGACAATAA ATTTAAAAAT
C. vomitoria 2   AATTAAACTA ATAATAAATT TATAAGCCAA AATAAAACTT AATACAATTA ATTTAAAATT

C. vicina 1      ATAATTTTTT TTAACTTATA AATTTAACAT TATTTAAATA AATAAAATTA GTAAACATGA
C. vomitoria 1   TATAAATTTT TTAATTTATT AATATAATAT TATT AAATA AATTAAATTA GTAAACATGA
C. vicina 2      ATAATTTTTT TTAACTTATA AATTTAACAT TATTTAAATA AATAAAATTA GTAAACATGA
C. vomitoria 2   TATAAATTTT TTAATTTATT AATATAATAT TATT AAATA AATTAAATTA GTAAACATGA

C. vicina 1      AAACCCTATT CTTAATGAAT TCATTATTAT ATTTAATAAA ATTAAACTAC TTCTAAAAAT
C. vomitoria 1   AAACCCTATT CTTAATGAAT TAAATCATTT TTAATAAAAA TTTAATTAAT AATATTAATT
C. vicina 2      AAACCCTATT CTTAATGAAT TGCCTGATAA AAAGGATTAC CTTGATAGGG TAAATCATGT
C. vomitoria 2   AAACCCTATT CTTAATGAAT TGCCTGATAA AAAGGATTAC CTTGATAGGG TAAATCATGT

C. vicina 1      AAATTCATTT AAATTAATA
C. vomitoria 1   TATTATTATT AATTTTTCT
C. vicina 2      AATAATATTA CATTCATTA
C. vomitoria 2   AATAATATTA CATTCATTA
```

Figure 9.4. Alignment of the 5' end of the control region for *C. vicina* and *C. vomitoria* consensus sequences. Two sequence types are presented – labelled 1 and 2. A vertical line divides the regions of similarity between the two types of control region (to the left) from the region showing distinct difference (to the right of the line).

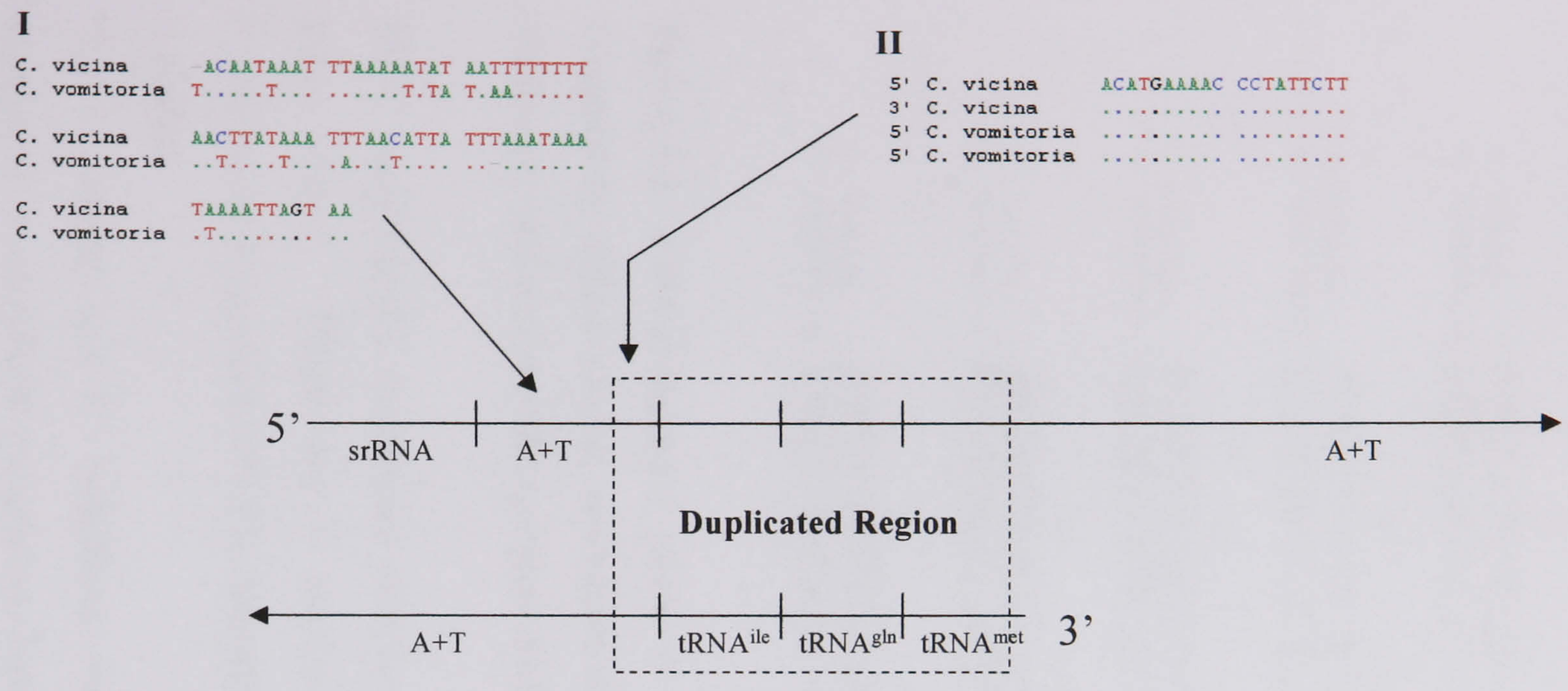
The vertical line in Figure 9.4, separates the similar region between type 1 and 2 from the region that differs.

After the vertical line the type 2 sequence is identical in both species whereas the type 1 sequence shows a high level of interspecific variation.

9.2.2 tRNA duplication within the control region

When examining the multiple amplicons produced with the SR-J-14612/TM-N-193 and SR-J-14761/TI-N-23 primer pairings it became clear that either the primers were binding in more than one location or that the samples showed length heteroplasmy for this region. After sequencing the 3' end of the control region the sequence of the tRNA coding regions was determined. On comparison with the 5' end of the type 2 sequences it became clear that the tRNA sequences were present at the 5' end too. There is therefore duplication of the tRNA coding regions $tRNA^{ile}$, $tRNA^{gln}$ and $tRNA^{met}$ at the 5' end of the type 2 control region. The arrangement of this duplicated region is presented in Figure 9.5.

Figure 9.5 also indicates the alternative binding sites of the two reverse primers TM-N-193 and TI-N-23. During the amplification of the external and nested regions the reverse primers will bind in two places – one in the $tRNA^{ile/met}$ coding region proper and one in the $tRNA^{ile/met}$ duplicated regions. This produces the 1500bp fragment and the small amplicons evident in Figure 9.3.



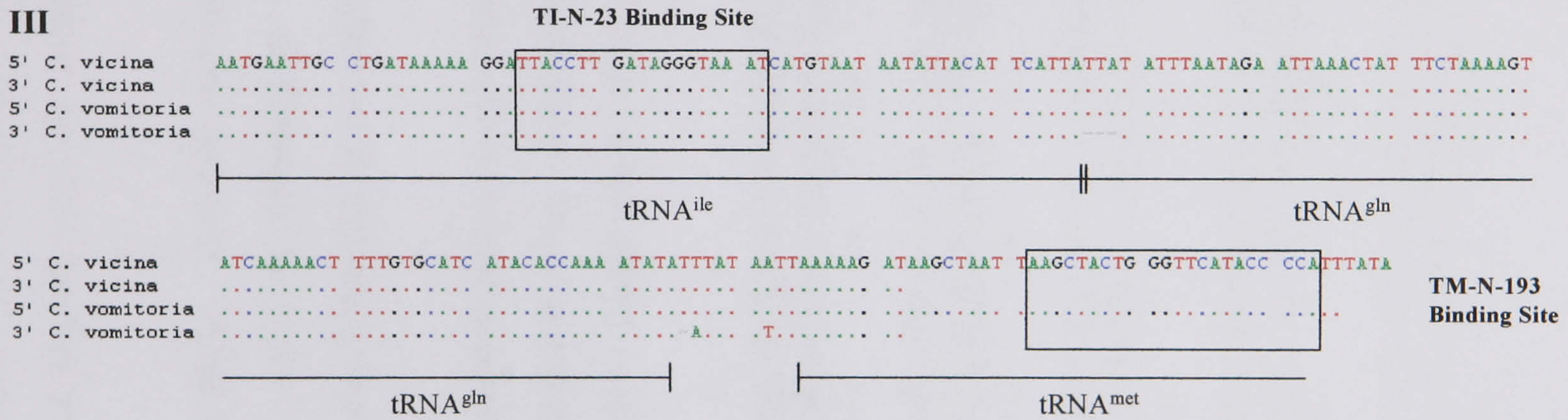


Figure 9.5. Layout of the mitochondrial control region in *C. vicina* and *C. vomitoria*. The flanking tRNA coding regions at the 3' end of the A+T rich region are duplicated at the 5' end. Dashed lines highlight the duplicated region. I – sequence of *C. vicina* and *C. vomitoria* partial control region. II – sequence of 19bp duplicated region. III – sequence of duplicated tRNA coding regions from both ends of the control region. Solid boxes surround reverse primer binding sites.

9.2.3 Small rRNA Region

As demonstrated in Figure 9.1, the forward primer SR-J-14612 is located within the srRNA coding region. Figure 9.6 shows the partial sequence for this region for both *C. vicina* and *C. vomitoria*. By comparison with the confirmed Dipteran srRNA sequences in GenBank, the 3' boundary of the srRNA was established for the two *Calliphora* species.

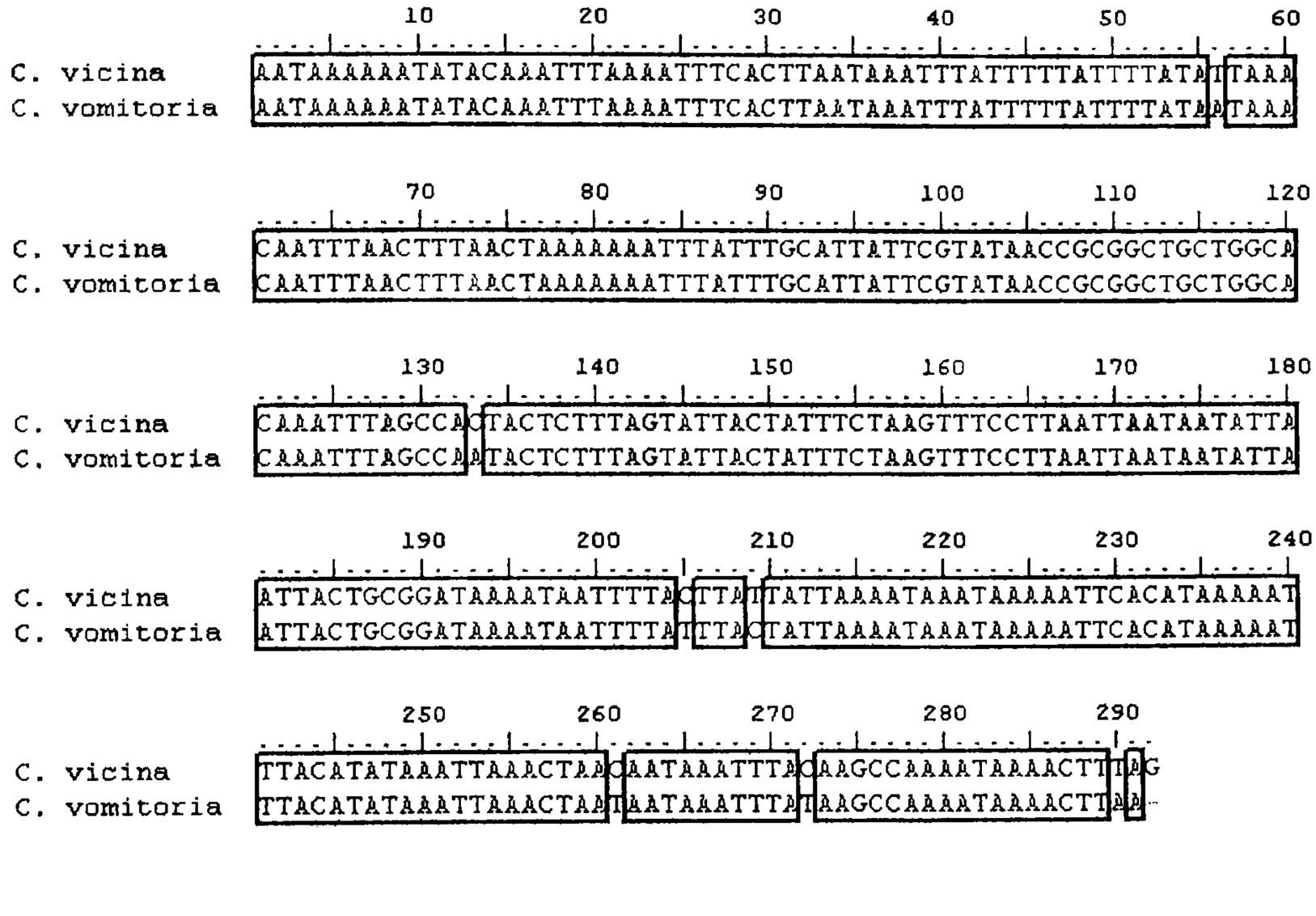


Figure 9.6. Partial sequence of the 3' end of the small rRNA region for *C. vicina* and *C.vomitoria*. Black outline surrounds identical bases. Base numbering is specific to this alignment only and not the position within the mitochondrial genome.

All *C. vicina* and *C. vomitoria* samples sequenced in this study matched the sequences given above. There are 8 nucleotide differences between the *C. vicina* and *C.vomitoria* sequences (97.3% identity between species). There was no intraspecific variation.

The *C. vicina* and *C. vomitoria* sequences for this partial srRNA region were deposited in GenBank (accession numbers DQ056742 and DQ056743 for *C. vicina* and *C. vomitoria* respectively). These are the first srRNA sequences for *C. vicina* and *C. vomitoria* to be deposited in GenBank.

When comparing the small rRNA sequence with those in GenBank, by using the NCBI BLAST facility, there were high identities with other Calliphoridae and

dipteran small rRNA sequences. The closest match was a *C. putoria* sequence (AF352790; for *C. vicina* 271/291 95% expect value e^{-126}; for *C. vomitoria* 277/289 95% expect value e^{-129}). There were similarities with *C. hominivorax* (AF260826; for *C. vicina* 267/292 91% expect value $4e^{-98}$; for *C. vomitoria* 267/290 92% expect value e^{-101}), *C. macellaria* (AF151385; for *C. vicina* 168/179 93% expect value $7e^{-63}$; for *C.vomitoria* 169/177 95% expect value $8e^{-75}$), *Dermatobia hominis* (Linnaeus 1781) (AY463155; for *C. vicina* 211/233 90% expect value $1e^{-67}$; for *C. vomitoria* 211/231 91% expect value $3e^{-71}$), *Lucilia eximia* Robineau-Desvoidy 1830 (AF151387; for *C.vicina* 167/179 93% expect value $2e^{-66}$; for *C. vomitoria* 166/177 93% expect value $1e^{-67}$) and *P. regina* (AF262957; for *C. vicina* 171/186 91% expect value $2e^{-63}$; for *C.vomitoria* 172/184 93% expect value $2e^{-69}$).

The nucleotide composition of this region is presented in Table 9.4 for both species. The level of A+T is 82% in *C. vicina* and 83.5% in *C. vomitoria*. Also presented in Table 9.4 are other regions (coding and non-coding) sequenced in this work on the mitochondrial control region.

Table 9.4. Nucleotide composition of the various coding and non-coding regions sequenced in this study.

	A	C	G	T	%A	%C	%G	%T
***C. vicina* small rRNA**	129	36	16	111	44	12.5	5.5	38
***C. vomitoria* small rRNA**	132	33	15	112	45	11.5	5	38.5
***C. vicina* tRNA^{ile}**	27	7	10	22	41	10.6	15	33.4
***C. vomitoria* tRNA^{ile}**	27	7	10	22	41	10.6	15	33.4
***C. vicina* tRNA^{gln}**	30	9	4	23	45.5	13.5	6	35
***C. vomitoria* tRNA^{gln}**	30	9	4	23	45.5	13.5	6	35
***C. vicina* A+T Region 'A'**	167	15	13	159	47.2	4.2	3.7	44.9
***C. vomitoria* A+T Region 'A'**	175	11	13	155	49.4	3.1	3.7	43.8
***C. vicina* A+T Region 'B'**	204	33	15	166	48.8	7.9	3.6	39.7
***C. vomitoria* A+T Region 'B'**	147	24	6	156	44.1	7.2	1.8	46.9
***C. vicina* A+T Region 'C'**	37	3	1	30	52.1	4.2	1.4	42.3
***C. vomitoria* A+T Region 'C'**	35	1	1	34	49.3	1.4	1.4	47.9

9.2.4 A+T rich Region C

There is a 71bp region between the small rRNA gene region and the duplicated region (labelled sequence 'I' in Figure 9.5). This region shows a high A+T ratio of 94.4% in the *C. vicina* consensus and 97.2% in the *C. vomitoria* consensus sequences (Table 9.4). This region was compared with the GenBank database using BLAST separately for each species. For the *C. vicina* sequence the last 21 nucleotides in the sequence match 21 nucleotides in the *C. megacephala* mitochondrial sequence (AF151386; 21/21 bp 100%; expect value = 0.15). This matching *C. megacephala* sequence is also located after the small rRNA coding region.

For the *C. vomitoria* sequence there is a 23 nucleotide match with a *P. regina* sequence (AF262957; 24/24bp 100%; expect value = 0.002). This *P. regina* sequence in GenBank is designated as "A+T rich region following the small rRNA".

For the purposes of this study, this region is nominated as A+T rich region C, to differentiate it from the other two regions, A and B, as named by Lessinger and Azeredo-Espin (2000).

The interspecific and intraspecific variation of this region is discussed in Section 9.2.8.

9.2.5 19bp Conserved Region

There is a duplicated region of 19bp (labelled 'II' in Figure 9.5) that is conserved between the species and is identical at both ends of the control region. At the 5' end of the control region, this 19bp stretch of nucleotides is located between the A+T rich region C and the duplicated tRNA coding regions.

9.2.6 tRNA regions

Sequencing of the regions flanking the 3' end of the A+T rich region provided the complete sequences for the *C. vicina* and *C. vomitoria* $tRNA^{ile}$, $tRNA^{gln}$ and a partial sequence for $tRNA^{met}$. The boundaries of these coding regions were established by comparison with the Calliphoridae tRNA sequences in GenBank. The sequences of these tRNA regions are presented in Figure 9.5 labelled sequence 'III'. These regions are part of the sequences deposited in GenBank with accession numbers AY894834

and AY894833 (*C. vicina* and *C. vomitoria* respectively). Until this study there were no *Calliphora* species tRNA coding regions within the GenBank database.

All *C. vicina* and *C. vomitoria* samples were identical to the sequences presented in Figure 9.5. There was no intraspecific variation in the tRNA regions sequenced. There were no interspecific differences found between *C. vicina* and *C. vomitoria* sequences. The percentage of nucleotide content is presented in Table 9.4. The A+T% for $tRNA^{ile}$ is 74.4%. When comparing the $tRNA^{ile}$ sequence with those in GenBank there was a 100% match (66/66 nucleotides; expect value $2e^{-28}$) with *C.chloropyga* (AY220133), *C. albiceps* (AY220131), *C. putoria* (AF352790) and *C. hominivorax* (AF260826).

The *C. vomitoria* $tRNA^{gln}$ sequence is three nucleotides shorter than the *C. vicina* sequence. The A+T ratio is higher in $tRNA^{gln}$ (80.5%) than $tRNA^{ile}$. When comparing the $tRNA^{gln}$ sequence with those in GenBank there was a 100% match (69/69 nucleotides; expect value $3e^{-30}$) with *C. chloropyga* (AY220133), *C.albiceps* (AY220131), *C. putoria* (AF352790), *C. hominivorax* (AF260826), *C. megacephala* (AF151386), *D. yakuba* (X03240) and *D. melanogaster* (U37541).

The only interspecific variation occurs in between the $tRNA^{gln}$ and $tRNA^{met}$ regions. There is an indel and two base substitutions. No intraspecific variation occurred in this region.

Due to the binding site of the external reverse primer (TM-N-193) being located within the $tRNA^{met}$ region only a partial sequence for this region was obtained. This $tRNA^{met}$ partial sequence was too short to interrogate the GenBank database successfully. However visual alignment with the $tRNA^{met}$ sequences of Calliphoridae indicated a 100% match with *C. chloropyga* (AY220133), *C. albiceps* (AY2201310), *C. putoria* (AF352790), *C. megacephala* (AF151386) and *C.hominivorax* (AF260826) $tRNA^{met}$ sequences.

9.2.7 A+T rich regions A, B and C

The amplification of the two separate regions of the A+T rich region was undertaken using the primers (CMEG A/SR-J-14612 and CMEG AR/TM-N-193) designed by Lessinger and Azeredo – Espin (2000). The amplicons produced are presented in Figures 9.7 and 9.9.

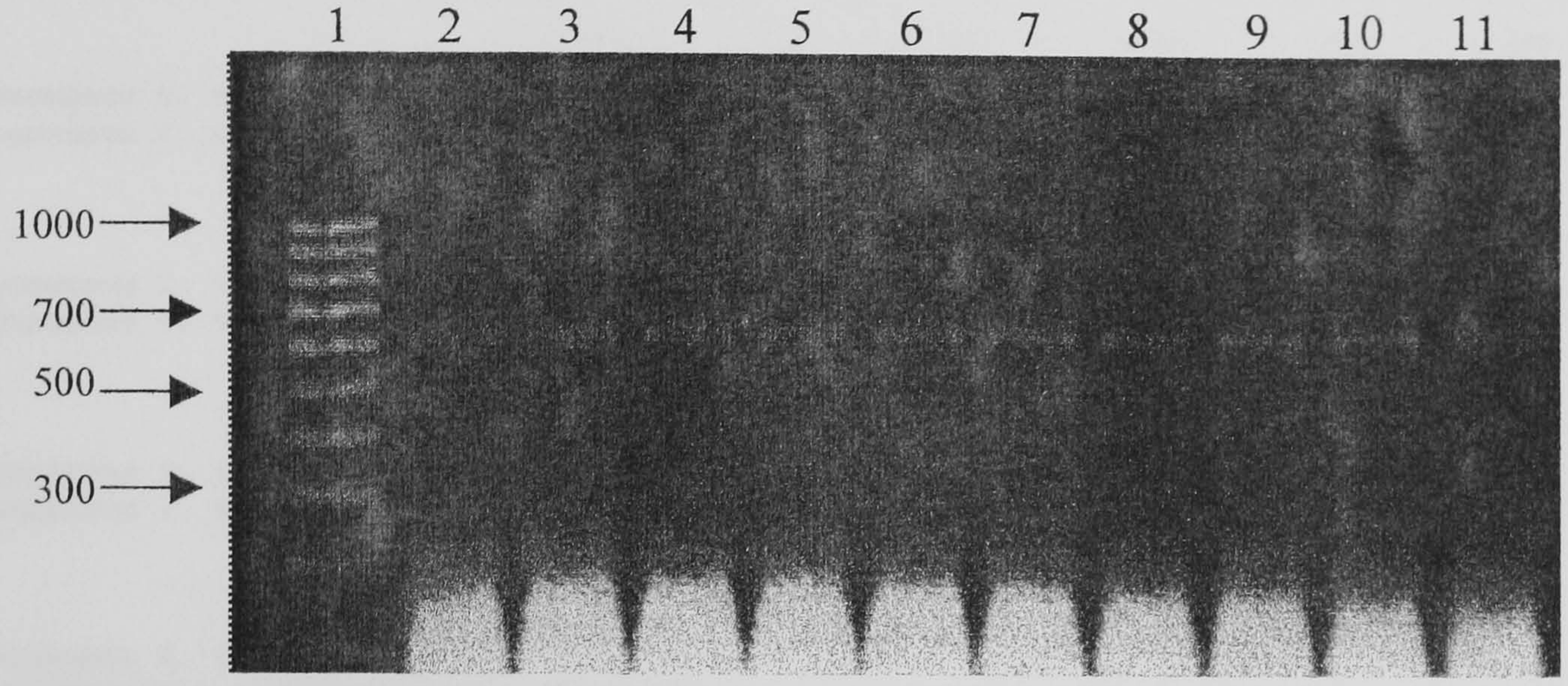


Figure 9.7. Amplicons produced for conserved region (A) using CMEG AR and TM-N-193 primers. Lane 1 contains 100bp DNA ladder. Lanes 2-6 contain examples of *C. vicina* amplicons. Lanes 7-11 contain examples of *C. vomitoria* amplicons.

The conserved region (A) of the A+T rich region appears to be over 600bp in size. There is no size variation in amplicons produced from these reactions both within and between samples. Consensus sequences for both species are presented in Figure 9.8. Note the conserved 19bp region shown to be present at both ends of the control region has been included in this Figure for alignment purposes. The consensus sequences for this region including the tRNA coding regions have been placed in GenBank, accession numbers AY894834 and AY894833 (*C. vicina* and *C. vomitoria* respectively).

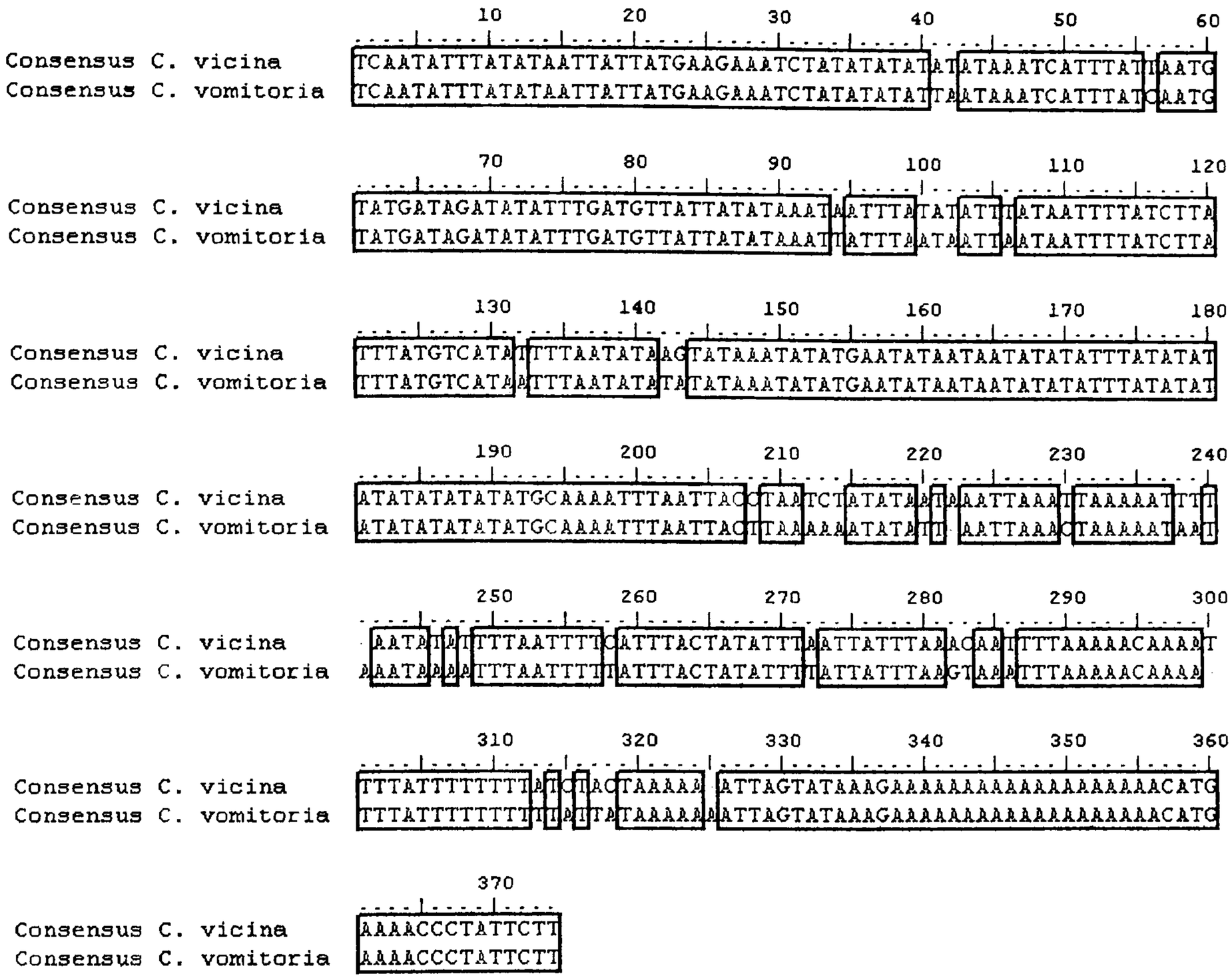


Figure 9.8. Alignment of *C. vicina* and *C. vomitoria* consensus sequences of the conserved control region. Black outline surrounds identical nucleotides. Base numbering is specific to this alignment only and not the position within the mitochondrial genome.

There are 34 differences between the species consensus sequences (9.1% variation). These are not all base substitutions as four indels are also present, at nucleotides 222, 241, 300 and 325bp. This part of the control region shows high levels of A and T residues (Table 9.4).

Both consensus sequences were compared to GenBank sequences using BLAST, and both had some identity with the equivalent region in other Calliphoridae. The *C.vicina* sequence had 171/208 nucleotide matches (82%; expect value $4e^{-07}$) with the *C. chloropyga* (AY220133) sequence; 76/86 nucleotide matches (88%; expect value $6e^{-06}$) with *C. megacephala* (AF151386) and 171/208 (82%; expect value $4e^{-07}$) with *C. putoria* (AF352790). The *C. vomitoria* sequence had 110/125 nucleotide matches (88%; expect value $2e^{-12}$) with the *C. chloropyga* (AY220133) sequence; 110/125

nucleotide matches (88%; expect value $2e^{-12}$) with *C. megacephala* (AF151386); 88/102 nucleotide matches (86%; expect value $3e^{-08}$) with *C. albiceps* (AY220131) and 110/125 (88%; expect value $2e^{-12}$) with *C. putoria* (AF352790).

The variable region (B) amplicons for *C. vicina* and *C. vomitoria* are over 1500bp. There is no size variation in amplicons produced from these reactions both within and between samples.

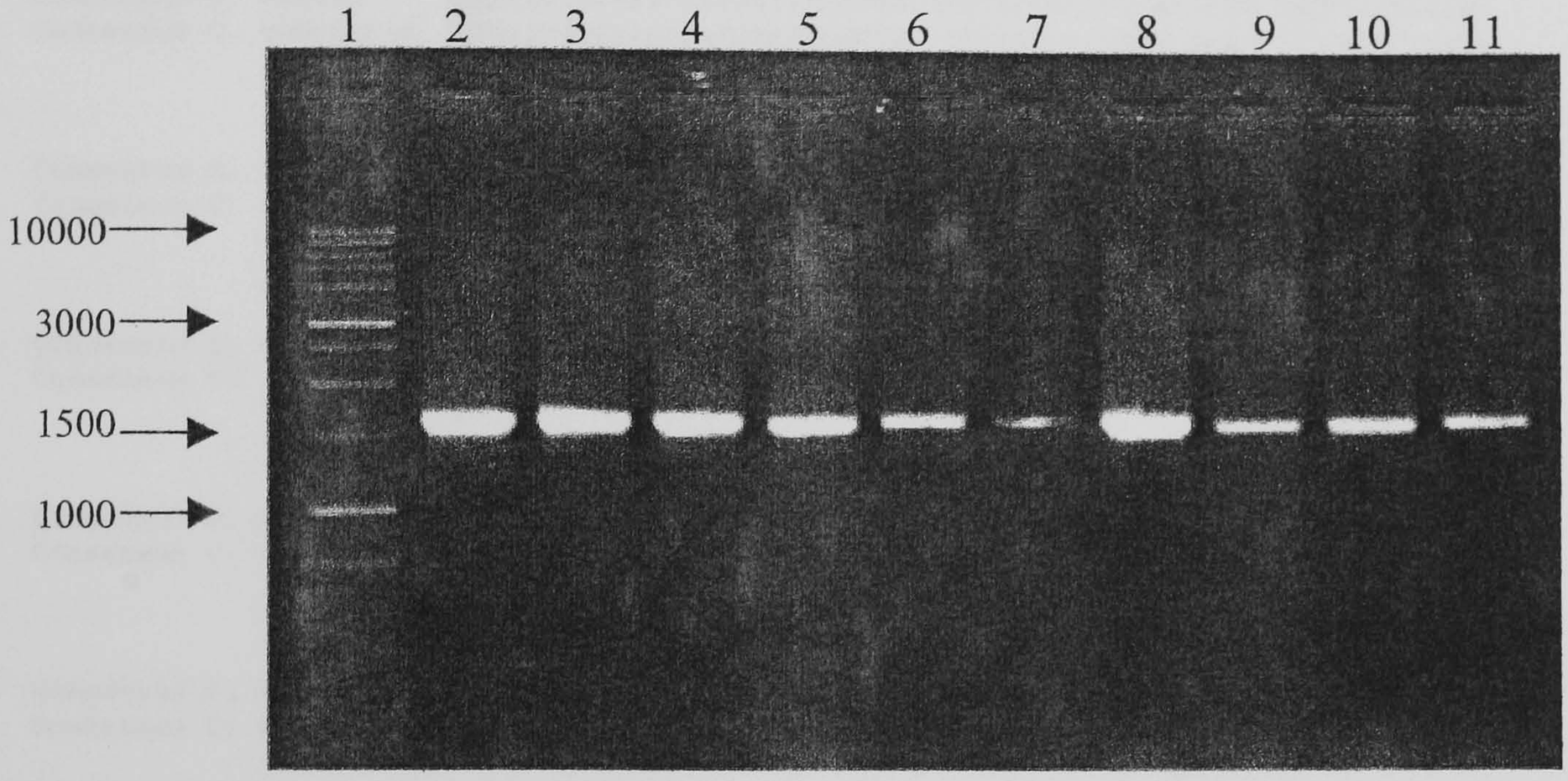


Figure 9.9. Amplicons produced for variable region (B) using CMEG A and SR-J-14612 primers. Lane 1 contains a 1KB DNA ladder. Lanes 2-6 contain examples of *C. vicina* amplicons. Lanes 7-11 contain examples of *C. vomitoria* amplicons.

The complete sequences for *C. vicina* and *C. vomitoria* amplified using SR-J-14612 and CMEG A are depicted in Figure 9.10. The forward primer is located in the srRNA coding region but as this region has been discussed previously (Section 9.2.3) this sequence has not been included in this Figure.

It is clear when using this primer pair that only type 1 control regions are amplified (the control region without tRNA duplication). Figure 9.10 includes the region designated in this study as A+T rich region C. When previously comparing the tRNA duplicated region in type 2 control regions (Figure 9.5) it was noted that there was a 19bp conserved region. This is also present in the type 1 control regions, located similarly after the 71bp A+T rich region C.

As mentioned in Section 9.2.1, A+T rich region B is highly variable between these two species. Figure 9.10 demonstrates that the sequences cannot easily be aligned; there are few conserved regions.

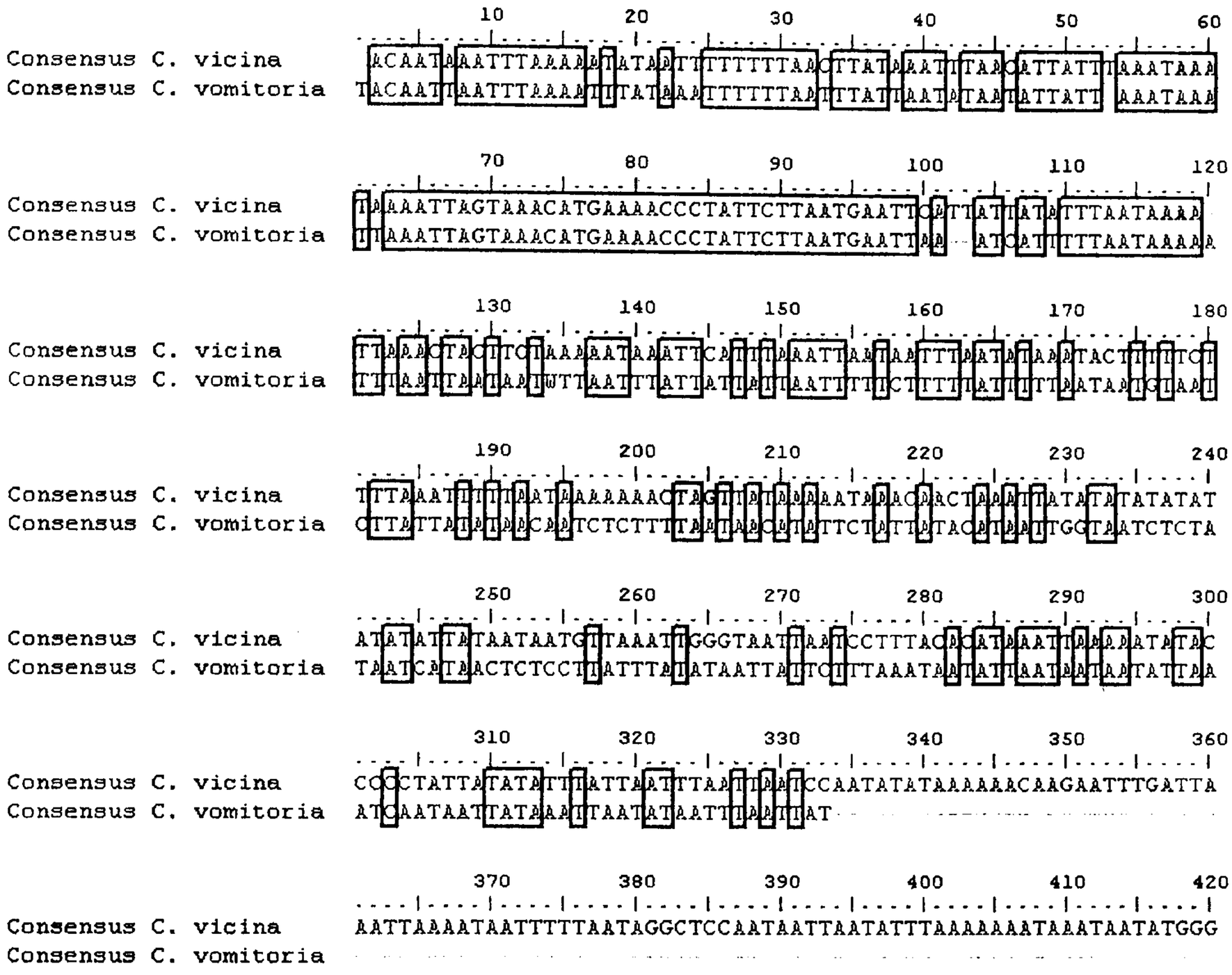


Figure 9.10. Alignment of *C. vicina* and *C. vomitoria* A+T rich B and C region sequences. Base numbering is specific to the presented sequence and not to the overall mitochondrial genome. Black outline surrounds identical nucleotides.

For A+T region B, there are 138 variable sites between *C. vicina* and *C. vomitoria* consensus sequences (57% variation. There are 103 nucleotides conserved between the two species (43% conservation). In comparison there are 59 conserved sites between *C. vicina* and *C. vomitoria* A+T rich region C consensus sequences (82% identity). The nucleotide composition of the A+T region B is presented in Table 9.4. The ratio of A+T together is 88.5% for *C. vicina* and 91% for *C. vomitoria*.

9.2.8 A+T rich Region A intraspecific variation

This part of the control region was amplified in 35 *C. vicina* and 37 *C. vomitoria* samples from varying populations. On examination of the alignment of all the samples (Appendix VII) the region has a relatively low level of intraspecific variation. Eight *C. vicina* individuals show one nucleotide substitution with the consensus sequence (0.27% variation) and three show two substitutions (0.54% variation). Three *C. vomitoria* individuals show one nucleotide differences to the consensus sequence (0.27% variation) and one has two nucleotide differences (0.54% variation). Appendix VII contains the allele frequencies for both species at loci containing variation.

An AMOVA was conducted on the two species separately and results presented in Tables 9.5 a and b.

Table 9.5a. AMOVA to assess variation between *C. vicina* populations for the A+T rich region A. df – degrees of freedom; SSq – sum of squares; MSq – mean of SSq; Est. Var. – estimated variation; Prob – probability value (p).

Source of Variation	df	SSq	MSq	Est. Var.	% Variation	Φ_{PT}	Prob
Between Populations	8	3.938	0.492	0.026	6%	0.0625	0.100
Within Populations	24	9.517	0.397	0.397	94%		
Total	32	13.455					

Table 9.5b. AMOVA to assess variation between *C. vomitoria* populations for the A+T rich region A. df – degrees of freedom; SSq – sum of squares; MSq – mean of SSq; Est. Var. – estimated variation; Prob – probability value (p).

Source of Variation	df	SSq	MSq	Est. Var.	% Variation	Φ_{PT}	Prob
Between Populations	6	0.791	0.132	0.000	0%	0.0000	0.550
Within Populations	29	4.014	0.138	0.138	100%		
Total	35	4.806					

The null hypothesis tested with these two separate AMOVA was that there was no variation in allele frequencies between populations. The probability values indicate that the null hypothesis can be accepted; there is no statistically significant variation between the populations for each species at this region of the mitochondrial genome. The Φ values are equivalent to Wright's F_{ST} values. For both species, these values are not significantly different from zero.

As the statistics indicated that there was no significant difference between populations, neighbour joining trees are not presented. For both species, the majority of populations would be grouped together. Mantel correlation coefficients for genetic and geographic distance are presented in Table 9.6.

Table 9.6. Mantel correlation coefficients and corresponding probability values for *C.vicina* and *C. vomitoria* population genetic and geographic distances for the A+T rich region A.

	SSx	SSy	SPxy	Rxy	p value
C.vicina	590045.938	0.000	-2.301	-0.257	0.890
C.vomitoria	722120.938	0.000	-0.847	-0.301	0.773

The Mantel correlation coefficients are not statistically significant at the 5% level and therefore indicate no relationship between geographic and genetic distance for *C.vicina* and *C. vomitoria* populations when using this part of the control region.

9.2.9 A+T rich Region C intraspecific variation

The total alignment of this region for all *C. vicina* and *C. vomitoria* samples is presented in Appendix VIII. Intraspecific variation is present in these sequences. There are three variable sites in *C. vicina* and six in *C. vomitoria*. This gives overall intraspecific variation of 4.22% for *C. vicina* and 8.45% for *C. vomitoria*.
The allele frequencies for each species are presented in Appendix VIII. There are no private alleles in *C. vicina* populations.

An AMOVA was conducted on both species to assess whether there were any genetic differences between the populations. Results are presented in Tables 9.7 a and b.

Table 9.7a. AMOVA to assess variation between *C. vicina* populations for the A+T rich region C. df – degrees of freedom; SSq – sum of squares; MSq – mean of SSq; Est. Var. – estimated variation; Prob – probability value (p).

Source of Variation	df	SSq	MSq	Est. Var.	% Variation	Φ_{PT}	Prob
Between Populations	10	6.325	0.633	0.125	33%	0.3341	0.020
Within Populations	23	5.733	0.249	0.249	67%		
Total	33	12.059					

Table 9.7b. AMOVA to assess variation between *C. vomitoria* populations for the A+T rich region C. df – degrees of freedom; SSq – sum of squares; MSq – mean of SSq; Est.

Source of Variation	df	SSq	MSq	Est. Var.	% Variation	Φ_{PT}	Prob
Between Populations	9	10.572	1.175	0.122	13%	0.1328	0.080
Within Populations	21	16.783	0.799	0.799	87%		
Total	30	27.355					

Var. – estimated variation; Prob – probability value (p).

For *C. vicina*, the alternative hypothesis that the population structure has caused genetic differentiation between the population is accepted. There is non-random mating occurring. For *C. vomitoria*, the test statistic is not significant at the conventional 5% level, however it should be noted that it is significant at 10% and therefore the *C. vomitoria* populations do show some differences albeit not statistically significant.

To assess whether these differences are related to the geographic distance between the populations, the genetic differences were calculated and the relationship displayed graphically (Figures 9.11 a and b). The Mantel correlation coefficient was also statistically tested (Table 9.8). As the AMOVA test statistic for *C. vomitoria* was significant at the 10% level, the *C. vomitoria* populations were included in the calculations.

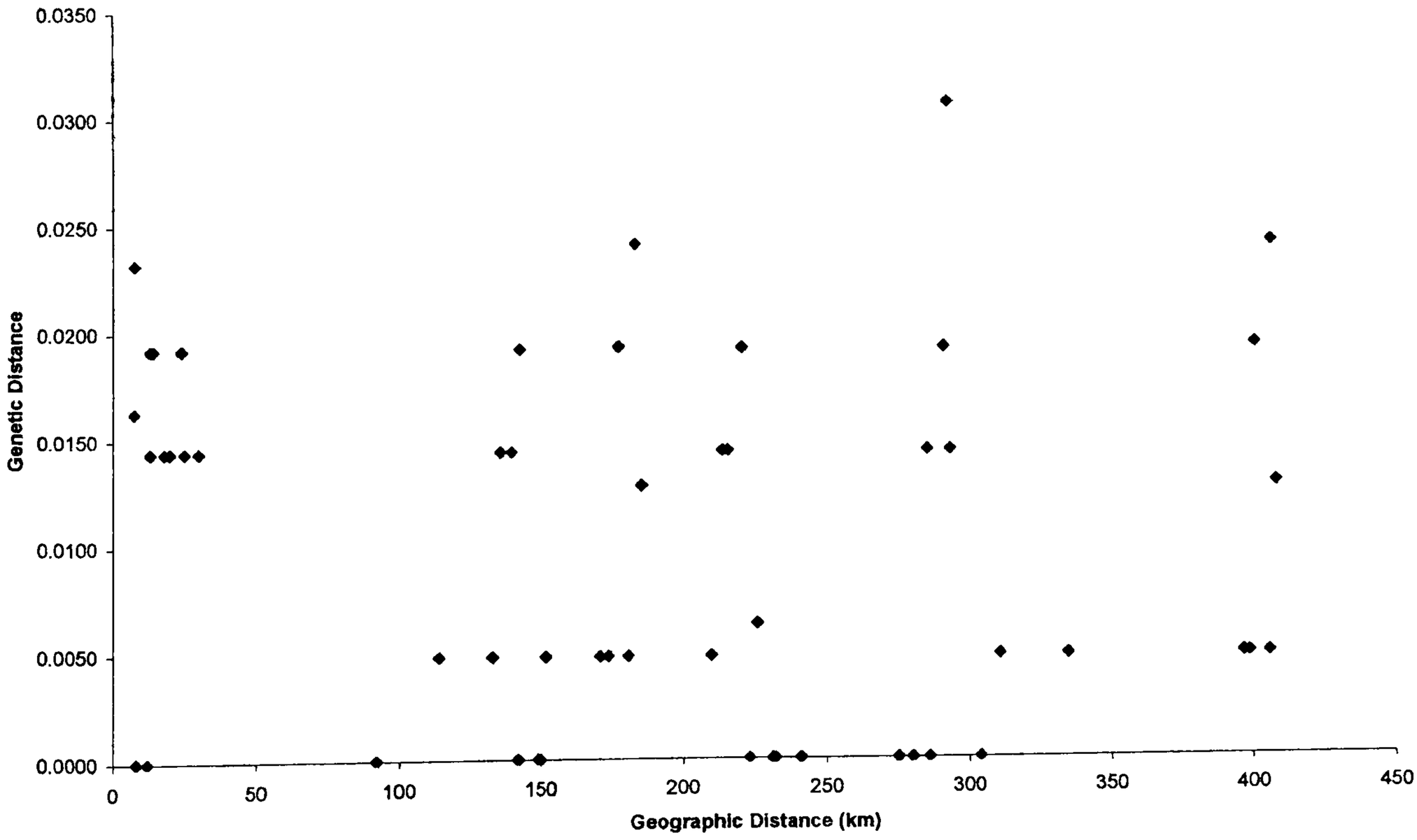


Figure 9.11a. Comparison of the geographic and genetic distances between *C. vicina* populations based upon the A+T rich 'C' region.

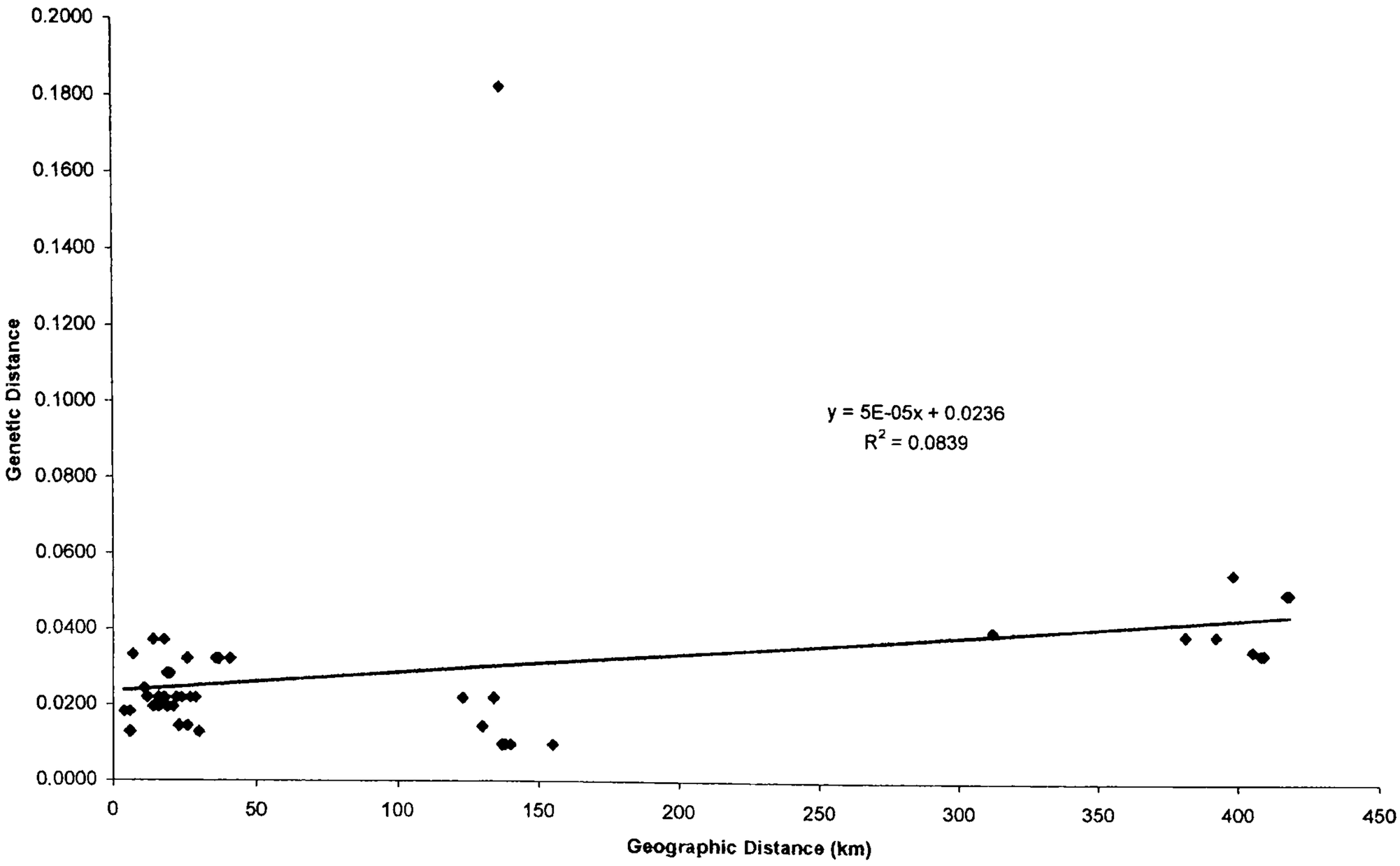


Figure 9.11b. Comparison of the geographic and genetic distances between *C. vomitoria* populations based upon the A+T rich 'C' region.

Table 9.8. Mantel correlation coefficients and corresponding probability values for *C. vicina* and *C. vomitoria* population genetic and geographic distances for the A+T rich region C.

	SSx	SSy	SPxy	Rxy	p value
C.vicina	855372.375	0.004	-5.540	-0.097	0.576
C.vomitoria	966612.313	0.030	49.120	0.290	0.036

The probability values of the Mantel coefficients indicated that there is no relationship between the geographic and genetic differences for *C. vicina* populations. However, for the *C. vomitoria* populations, there appears to be a statistically significant positive relationship between genetic structure and geographic distance.

Neighbour joining trees were constructed for both species (Figures 9.12 a and b).

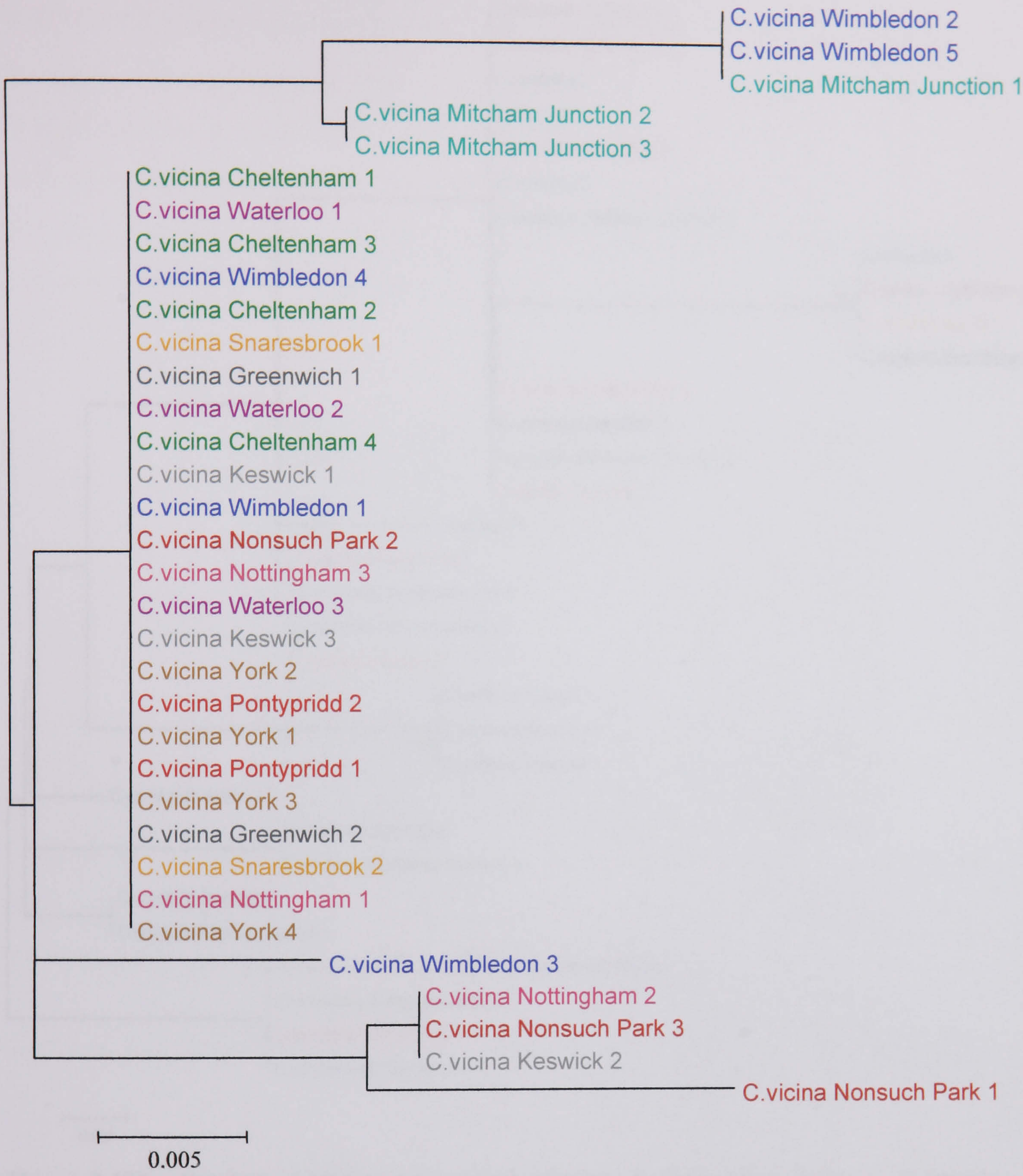


Figure 9.12a. Neighbour joining tree based upon A+T rich region C for *C. vicina* populations. Numbers indicate % confidence after bootstrapping with 1000 replicates.

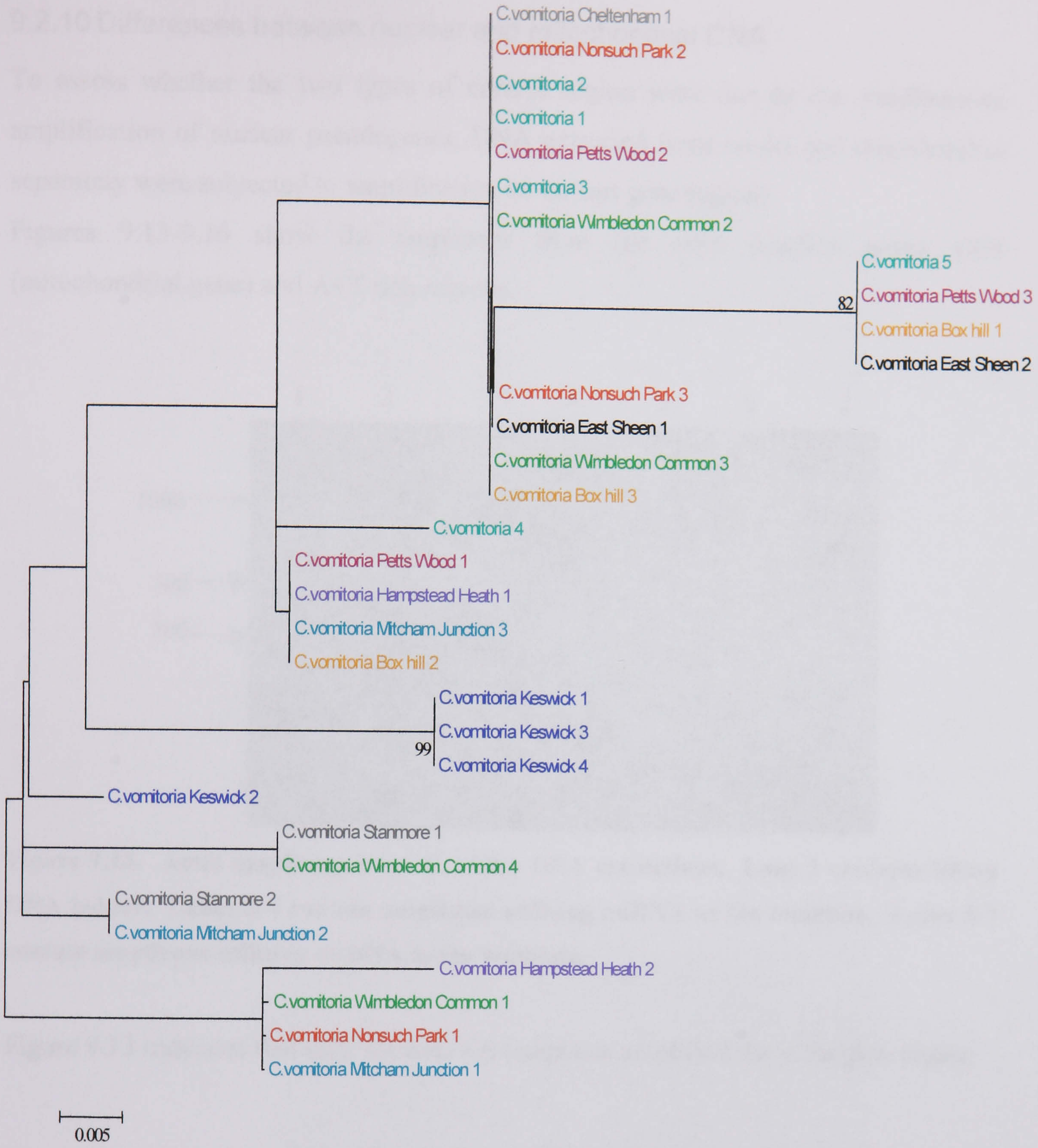


Figure 9.12b. Neighbour joining tree based upon A+T rich region C for *C. vomitoria* populations. Numbers indicate % confidence after bootstrapping with 1000 replicates.

The trees highlighted the similarities between the *C. vicina* populations as the majority of samples are grouped together. Those that have separate topology have low confidence values after bootstrapping. Conversely, there are distinct groupings of samples in the *C. vomitoria* tree and the confidence values are much higher. The tree shows a grouping of some Keswick samples, with a high confidence value.

9.2.10 Differences between nuclear and mitochondrial DNA

To assess whether the two types of control region were due to the simultaneous amplification of nuclear pseudogenes, DNA extracted from nuclei and mitochondria separately were subjected to amplification of various gene regions.

Figures 9.13-9.16 show the amplicons from the actin (nuclear gene), COI (mitochondrial gene) and A+T rich regions.

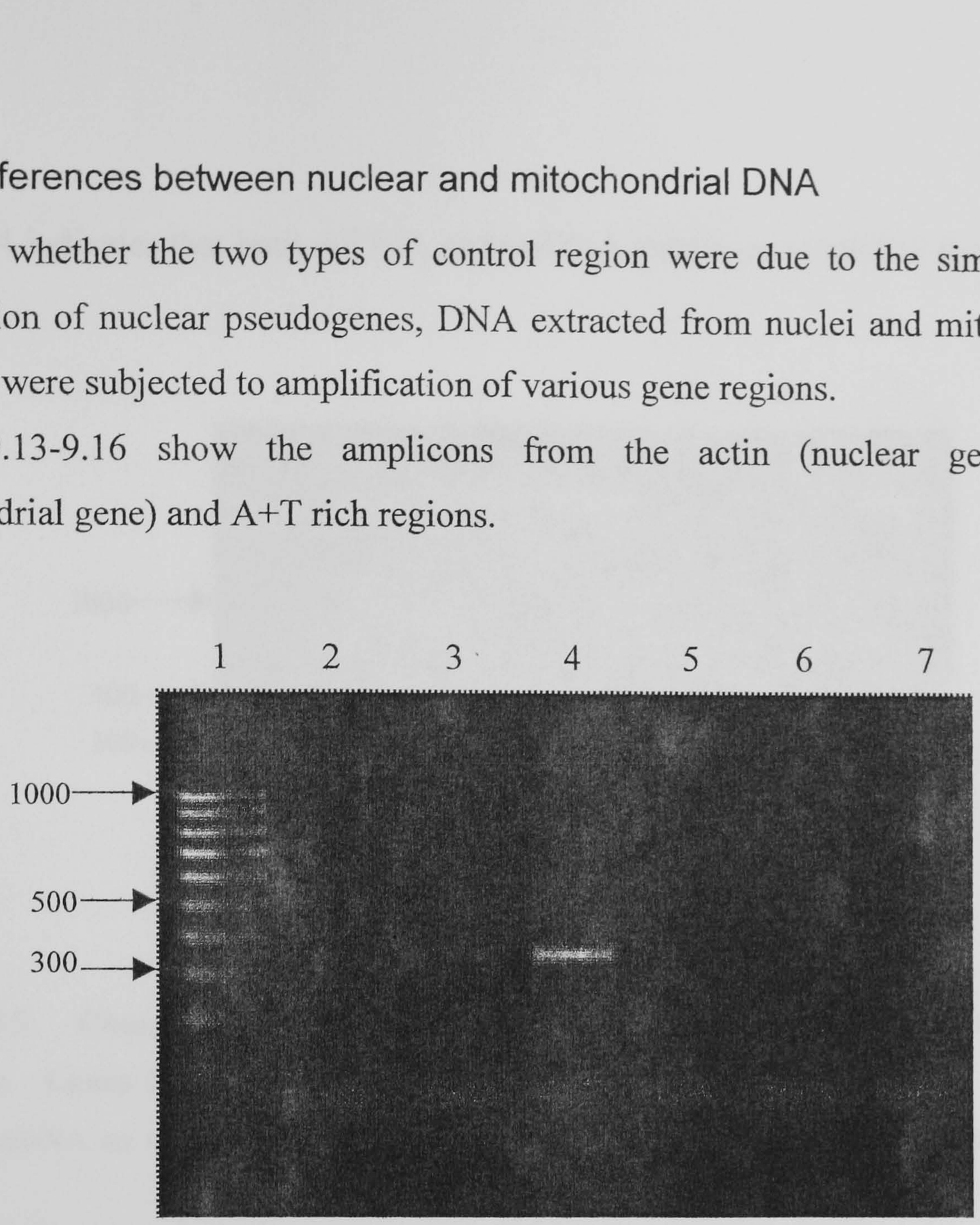


Figure 9.13. Actin amplicons from *C. vicina* DNA extractions. Lane 1 contains 100bp DNA ladder. Lanes 2-4 contain amplicons utilising nuDNA as the template. Lanes 5-7 contain amplicons utilising mtDNA as the template.

Figure 9.13 indicates that only the nuDNA templates amplified the actin gene region.

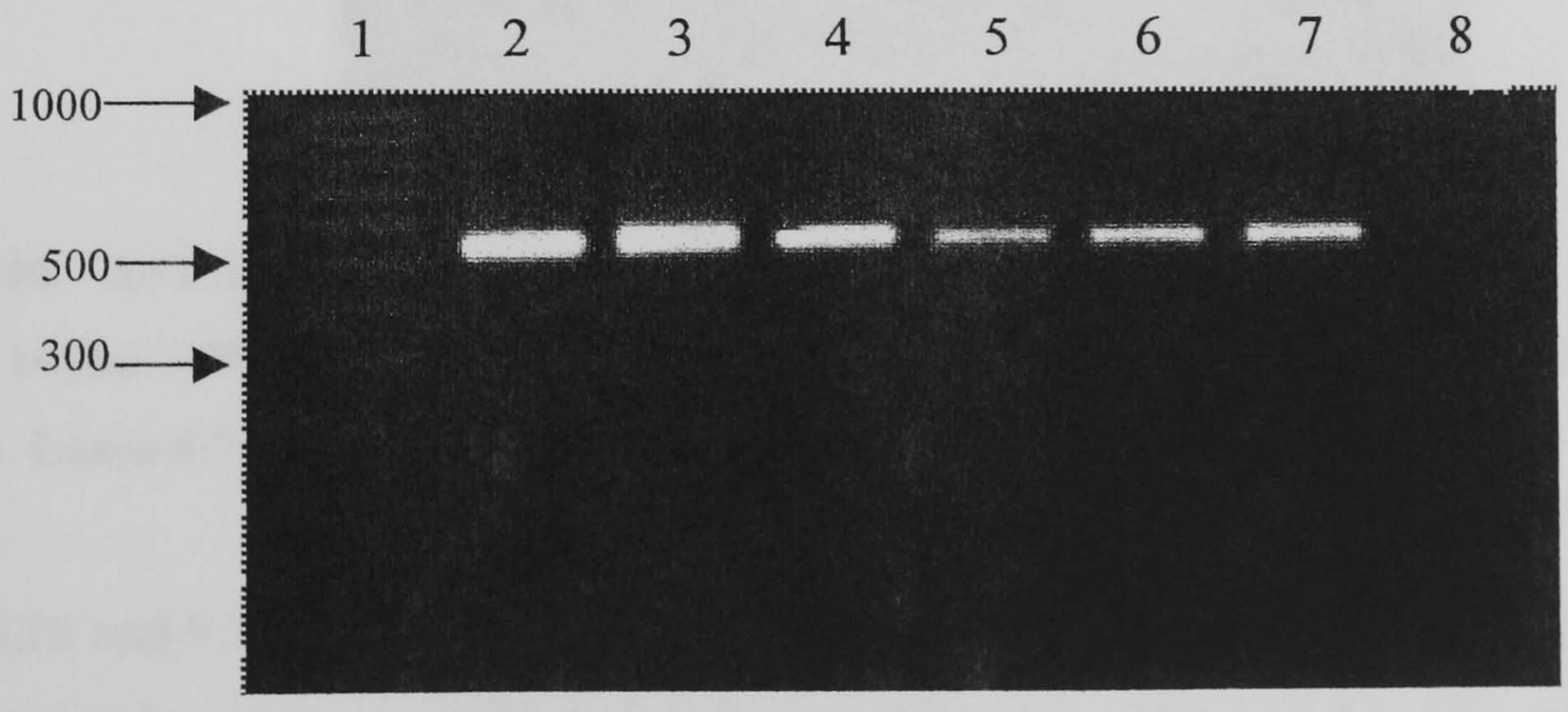


Figure 9.14. COI amplicons from *C. vicina* DNA extractions. Lane 1 contains 100bp DNA ladder. Lanes 2-4 contain amplicons utilising nuDNA as the template. Lanes 5-7 contain amplicons utilising mtDNA as the template. Lane 8 is negative control.

Figure 9.14 indicates that both nuDNA and mtDNA templates amplified the COI gene region.

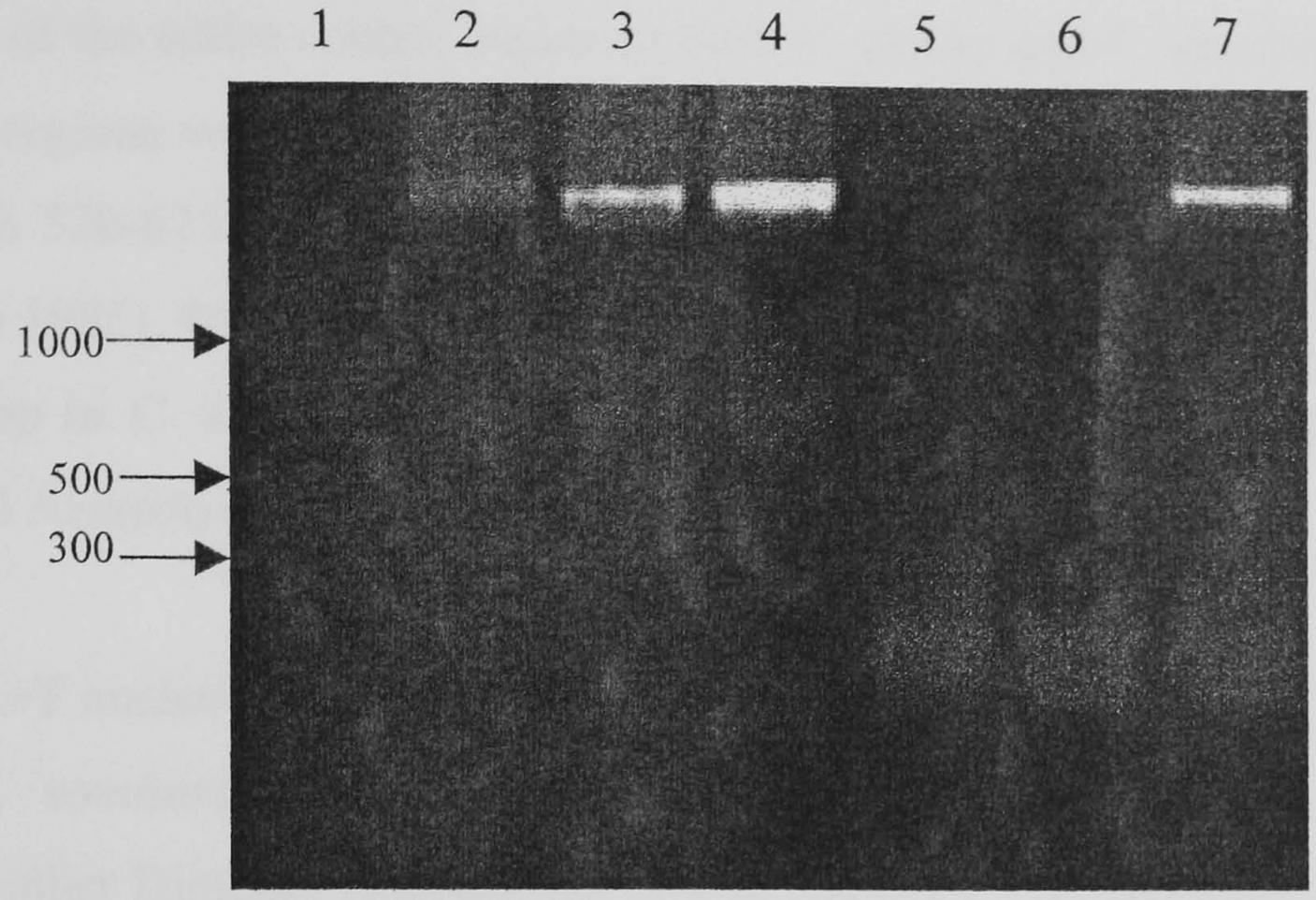


Figure 9.15. Complete A+T rich control region amplicons from *C. vicina* DNA extractions. Lanes 1 and 9 contain 100bp DNA ladder. Lanes 2-4 contain amplicons utilising nuDNA as the template. Lanes 5-7 contain amplicons utilising mtDNA as the template.

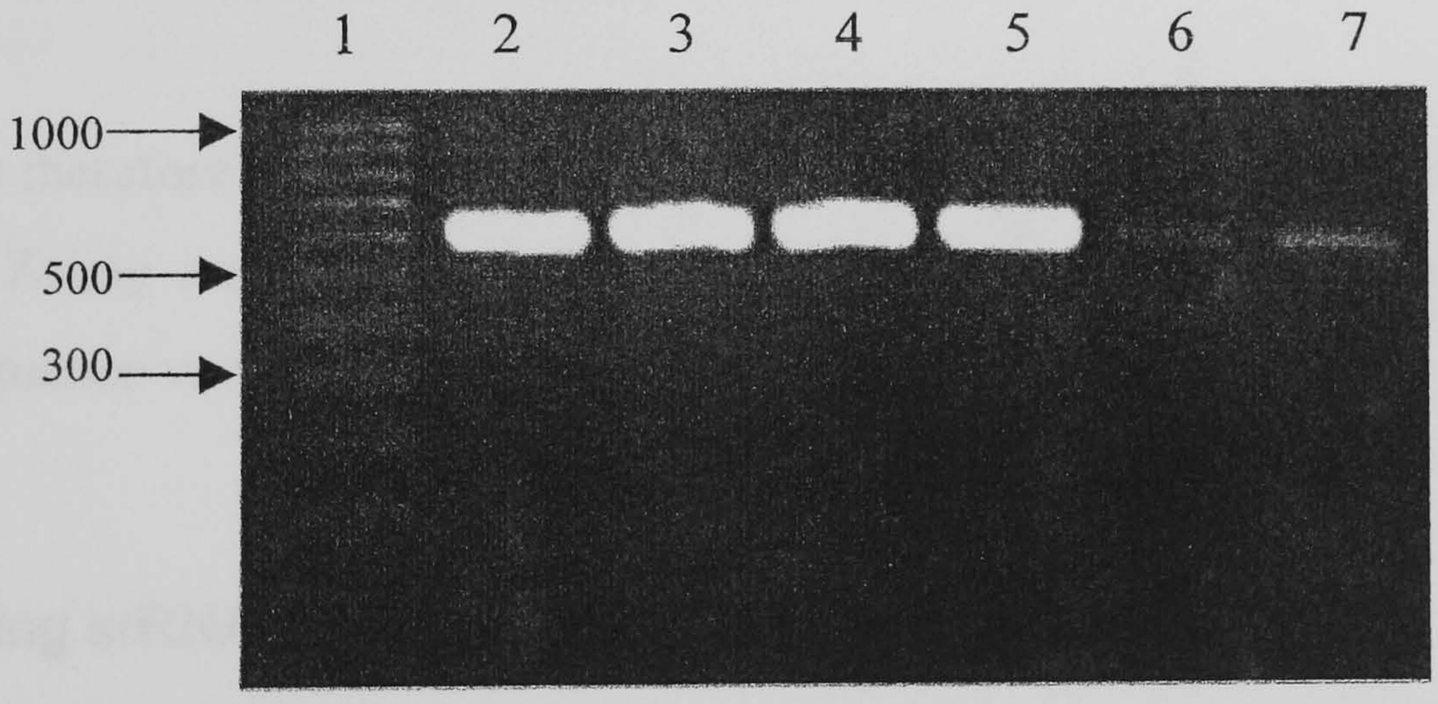


Figure 9.16. A+T rich region 'A' amplicons from *C. vicina* DNA extractions. Lane 1 contains 100bp DNA ladder. Lanes 2-4 contain amplicons utilising nuDNA as the template. Lanes 5-7 contain amplicons utilising mtDNA as the template.

Figures 9.15 and 9.16 indicate that both nuDNA and mtDNA templates amplified both types of control region, as multiple banding is present in Figure 9.15, although only one out of three mtDNA samples worked.

9.3 Discussion

9.3.1 Size and nucleotide composition of the control region

Amplification of the entire control region in both *C. vicina* and *C. vomitoria* samples indicated two regions were present with lengths of around 2000 and 1500bp. This is compared with 520-625bp for *Anopheles* species, 1077bp for *D. yakuba* (Clary and Wolstenholme 1985), 4601bp for *D. melanogaster* (value quoted in Zhang and Hewitt 1997b), 1178bp in *C. macellaria,* 1222bp in *C. megacephala*, 1130bp in *L. eximia* (Lessinger and Azeredo-Espin 2000).

The ratio of A+T nucleotides within the total control region is 91.6% for *C. vicina* and 93.8% for *C. vomitoria*. These high values are consistent with those already calculated for other Diptera. Brehm *et al.* (2001) and Clary and Wolstenholme (1985) found levels of 93% in *Drosophila obscura* and *D. yakuba* respectively. Junqueira *et al.* (2004) calculated 89.5% for *C. chloropyga* and values of 91% for *C.hominivorax*, 87% for *C. macellaria*, 88.5% for *C. megacephala* and 90.5% for *L. eximia* were noted by Lessinger and Azeredo-Espin (2000). The highest value is 96% in *A. mellifera* and *D. melanogaster* (Zhang and Hewitt 1997b).

There appears therefore to be a mutation pressure towards the A and T residues within this region. Zhang and Hewitt (1997b) postulated that this pressure will actually reduce the sequence variability and limits its use as a population marker.

9.3.2 Flanking srRNA and tRNA coding regions

Due to the location of the primer binding sites the sequences for srRNA and $tRNA^{ile}$, $tRNA^{gln}$ and partial $tRNA^{met}$ could be established for *C. vicina* and *C. vomitoria.* These were the first *Calliphora* entries into GenBank for these coding regions.

There were high identities between the species. For the srRNA region there was only a 2.7% difference between the *C. vicina* and *C. vomitoria* sequences which is lower than other coding regions sequenced in this work (COI – 4.1%; XDH – 6.7%). The A+T ratio was 82% for *C. vicina* and 83.5% for *C. vomitoria*, lower than that of the non-coding A+T rich regions (which ranged from 88.5%-97.2% depending upon species and region). Flook and Rowell (1997) found an average of 72.5% A+T (67.4-

77.3%) for 38 orthopteran species. Clary and Wolstenholme (1985) note a level of 79% for *D. yakuba*. Lessinger and Azeredo-Espin (2000) provide an average value of 82% for the srRNA for the Calliphoridae in their work. Junqueira *et al.* (2004) found a level of 76.9% for *C. chloropyga* srRNA.

The tRNA coding regions sequenced in this work also showed lower A+T ratios. The $tRNA^{ile}$ region was 74.4% for both species as there was no interspecific variation. The first three bases of the *C. vicina* $tRNA^{gln}$ sequence are not present in the *C.vomitoria* sequence. It is common in the mitochondrial genome that the beginning and ends of coding regions overlap. Junqueira *et al.* (2004) showed after sequencing the complete *C. chloropyga* genome that there were 13 incidences of coding regions overlapping. This could be occurring with the *C. vomitoria* $tRNA^{ile}$ and $tRNA^{gln}$ as the last three bases of the $tRNA^{ile}$ are 'TTA' and these are the three nucleotides extra to the *C. vicina* $tRNA^{gln}$ sequence. Assuming the regions overlap, the identity between the two species' $tRNA^{gln}$ sequence is noted as 100%.

For the tRNA genes of the cockroach (*P. fuliginosa*) and grasshopper (*Orthetrum triangulare*), Yamauchi *et al.* (2004) found sequences of 63-71bp, similar to the lengths of the tRNA coding regions sequenced for these *Calliphora* species.

No intraspecific variation was found in any of the tRNA regions, which indicates the sequences are under functional constraint due to the importance of these coding regions.

9.3.3 Control region tRNA duplication

Gel pictures of the control region included smaller amplicons that could not initially be explained. Sequencing of the 5' end of the longest fragments provided no explanation for the presence of smaller fragments. There did not appear to be other locations for the reverse primer to bind. Examination of the *D. yakuba* control region (Clary and Wolstenholme 1985) also gave no indication of an alternative binding site (although it was appreciated that the *D. yakuba* A+T rich region is shorter than the *Calliphora* fragments amplified here). It was initially decided that these small amplicons were PCR anomalies or perhaps located elsewhere in the mitochondrial or the nuclear genome until the small amplicons were sequenced. This indicated that the

reverse primers were indeed binding in two places. After sequencing the 3' end of the longer fragments and establishing the *Calliphora* sequence of $tRNA^{ile}$, $tRNA^{gln}$ and partial $tRNA^{met}$ by comparison with GenBank, it became clear that duplication of these genes was present. This finding coincided with the publication of the results of Lessinger *et al.* (2004) that duplication of complete $tRNA^{ile}$ and partial $tRNA^{gln}$ sequence exists in *Chrysomya* species.

Lessinger *et al.* (2004) note that of the Insecta control regions sequenced thus far only *Chrysomya* species show tRNA duplication. This study now indicates that *C. vicina* and *C. vomitoria* contain mitochondrial genome molecules that show tRNA duplication. *Chrysomya albiceps*, *C. megacephala* and *C. chloropyga* only contain partial $tRNA^{gln}$ regions whereas *C. vicina* and *C. vomitoria* show complete $tRNA^{gln}$ regions. The presence of partial tRNA sequences indicates that the 5' tRNAs are the duplicated psuedogenes that have been transposed from the original 3' location. It cannot be assumed however that they are all non functional, indeed Lessinger *et al.* (2004) propose that the *Chrysomya* $tRNA^{ile}$ duplicated gene is functional, although this does not appear to be based on experimental observations.

Both the *Chrysomya* species and *Calliphora* species also show duplication of a short stretch of nucleotides. For both genera, this is a 19bp stretch. These two sequences show no identity between genera. The *C. vicina* and *C. vomitoria* sequences show 100% match between species and also between the duplicated 5' and the 3'stretch indicating this region is highly conserved. For *C. albiceps* this 19bp region duplicated region also matches completely with the region located before the tRNA coding regions flanking the 3' end of the A+T rich region. The corresponding regions in *C. chloropyga* and *C. megacephala* show slight changes.

To demonstrate this control region layout in both *Chrysomya* and *Calliphora* species, the duplication event must have occurred before diversification but after Brachycera split into Calyptratae and Acalyptratae as Drosophila species do not demonstrate this tRNA duplication (Clary and Wolstenholme 1985, Zhang *et al.* 1995). The discovery that the *C. vomitoria* duplicated region matches *C. vicina* duplicated region but that the *C. vomitoria* inter-tRNA coding regions do not match *C. vicina* exactly implies a previous duplication event in the ancestor to these species and a subsequent mutational event in the *C. vomitoria* tRNA coding regions.

The duplication of tRNA genes in the mitochondrial genome is not a new phenomenon as Zhang and Hewitt (1997b) noted that the flanking gene order of the control region varies in different Insecta. Indeed rearrangement of tRNAs within gene clusters has been reported in vertebrates too. If comparison is made between the mitochondrial genomes of humans and *D. yakuba* (Anderson 1981; Clary and Wolstenholme 1985) it can be seen that the gene order varies, when according to the theory of the origins of mitochondria, these must have once been identical. Therefore tRNA regions seem prone to duplication/transposition events.

However, two factors are contrary to the theory of a duplication occurring in a common ancestor to *Chrysomya* and *Calliphora*. The first, as noted by Lessinger *et al.* (2004) is that *C. megacephala* $tRNA^{ile}$ has two nucleotide substitutions from *C.albiceps* and *C. chloropyga* $tRNA^{ile}$ sequence. These substitutions are also present in the *C. megacephala* duplicated region. This implies that either duplication occurred after division within the *Chrysomya* genus or that the regions of nucleotide substitution have higher tendency to mutate and that the two nucleotide substitutions happened independently at opposite ends of the *C. megacephala* control region.
The other contradicting factor is that the *L. eximia* control region sequenced by the Lessinger *et al.* research group does not show tRNA sequence duplication. As indicated by the COI tree (Figure 6.8) *Calliphora* and *Lucilia* are more closely related genetically than *Calliphora* and *Chrysomya*. It is therefore likely that *Calliphora* and *Lucilia* diverged from each other more recently than from *Chrysomya.*

As discussed at the start of this Chapter, length heteroplasmy has been shown to be common in a variety of species. In most cases this is due to the presence of repeat sequences within the control region thought to be caused by slippage during replication. The prominent cause of the length variation of the A+T rich region within individuals from this study for *C. vicina* and *C. vomitoria* appears to be two types of control region – one with and one without tRNA duplication. The presence of two types of control region is not simply the insertion of the duplicated tRNA genes and 19bp conserved nucleotide stretch, as it is the smaller of the two control region types that contains this region.
As the whole control region of either type were not sequenced in this study it is not

possible to conclude whether the tandem repeated sequences discussed at the beginning of this Chapter are included in *C. vicina* and *C. vomitoria* control regions.

The presence of length heteroplasmy in insect control regions has been reviewed by Zhang and Hewitt (1997b). They note that even though the difference in control region size obviously relates to an overall change in total mitochondrial genome length, that there is not always a skew toward the shorter sized molecules. In this work, the longer control region fragments appeared to be the more plentiful within most individuals (this was however just assessed by eye from electrophoresis gel pictures and not objectively measured). Conversely, Hale and Singh showed a definite tendency for shorter molecules in *D. melanogaster* (Hale and Singh 1991).

The presence of these two types of control region is problematic for a forensic entomologist, as use of primers that bind to flanking tRNA genes will amplify both types and will bind to the duplicated region requiring separation by electrophoresis or cloning before sequencing. Even if reverse primers that bind outside of the $tRNA^{ile}$, $tRNA^{gln}$ and $tRNA^{met}$ trio are used, for instance in the NADH dehydrogenase 2 gene, they will still amplify both types of control region and produce long fragments that cannot be sequenced in one run.

The alternative to this is to use primers that either have a forward primer that binds within the $tRNA^{met}$ and a reverse in $tRNA^{ile}$ – thus amplifying only the type 2 control region or use a primer that binds within the control region and a other that binds in the flanking genes. The latter was done to amplify the regions A and B as designated by Lessinger and Azeredo-Espin (2000). Electrophoresis pictures of the amplification products showed only one amplicon. After consequent sequencing it was shown that only type 1 control region was being amplified. This indicated that the conserved block V as discovered by Lessinger and Azeredo-Espin (2000) upon which the CMEG A and CMEG AR primers are designed is not present in type 2 control regions.

Whilst changing primer combinations will produce a single amplicon, for ease of sequencing analysis it should be noted that more than one primer combination should be used when studying the control region of a species not previously examined, to assess for heteroplasmy and gene duplication.

9.3.4 A+T rich regions A and B

The A+T rich region A was also named the conserved region by Lessinger and Azeredo-Espin (2000) due to the presence of eight conserved sequence blocks. The forward primer to amplify this region was based in CSB V. On examination of the regions of the Calliphoridae sequences in GenBank that had some identity with the *C.vicina* and *C. vomitoria* sequences, it became evident that they matched the first four conserved sequence blocks (CSBs I-IV). CSB I (a poly-A stretch) was also commented on by Zhang and Hewitt (1997b) as a structural element present in Diptera and Orthoptera. It has been postulated that this is maybe involved in the control of transcription or replication.

The region is also relatively conserved in sequence length between *C. vicina* and *C.vomitoria* but less so in DNA sequence as 34 interspecific differences were noted. This is greater variation than for COI and XDH and could therefore be used to distinguish between the two species. However due to the high level of A and T residues in this region (92% in *C. vicina* and 93% in *C. vomitoria*) there is a limitation to the number of restriction enzymes that can be used. Only seven cut the *C. vicina* sequence in total and only eight cut the *C. vomitoria* sequence, located using BioEdit software. Three of these enzymes (*Ase*I, *Apo*I and *Dra*I) cut many times thus creating small fragments. *Ssp*I and *Mbo*II cut in the same place in both species. *Pac*I only cuts in *C. vomitoria* and therefore would not distinguish *C. vicina* digested from undigested. The only two possible enzymes are *Psi*I, which cuts each species only once but in different locations or *Tsp*II, which cuts *C. vomitoria* 3 times and *C. vicina* twice. Assessing some of the other species for which the A+T rich region A has been deposited in GenBank, also indicated that the number of restriction enzymes is limited. Enzymes could distinguish between the species, although more than one enzyme was required.

The region was not useful for differentiating between populations, as the level of intraspecific variation was not only low but the between population variation was shown to be not statistically different to the within population variation. This could be due to the low sample numbers used in this section of work. The low level of variation was shown to be unrelated to geographical distance between the populations.

The Φ values are zero for *C. vomitoria* and close to zero for *C. vicina*, this indicates that the populations have nearly identical allele frequencies.

Sequencing of the A+T rich region B demonstrated that the duplicated 19bp stretch of nucleotides transposed along with the tRNA coding regions was also present in the type 1 control region. There are several possibilities for the presence of this region. The whole duplicated region, including the tRNA pseudogenes, was present originally in type 1 and the tRNA regions were transposed out of the 5' end at a later date. It could be that the regions of the 3' end of the control region were transposed independently – the 19bp stretch at a separate occasion to tRNA genes. Alternatively the 19bp conserved region could have originally been at the 5' end and was transposed to the 3' end between the A+T rich region and the tRNA coding regions. This last reason seems the most plausible, as there are 11 nucleotides upstream and 8 nucleotides downstream surrounding the 19bp region at the 5' end that are also conserved between species and the two types of control region. It seems more likely that this was a 37bp conserved region, present in the *C. vicina* and *C. vomitoria* common ancestor and at some point before the two species divided, 19bp of this region was transposed to the tRNA coding region at the 3' end. However, without more information on the mechanism for mitochondrial gene transfer and other Calliphoridae sequences it is difficult to reach a conclusion.

The A+T rich region B was also called the variable region by Lessinger and Azeredo-Espin (2000). The researchers found that this domain was difficult to align for the Calliphoridae that they sequenced. They found that only the *Cochliomyia* species could be aligned with a high degree of confidence.

The level of variability between *C. vicina* and *C. vomitoria* is the highest of all the gene regions examined within this work. As this is not a coding region the high number of differences between the species is not that surprising as there is no protein structure limitations on this region. Region A was less variable due to the conserved blocks thought to be associated with the origins of replication of the two mitochondrial strands.

9.3.5 A+T rich region C

This stretch of nucleotides was apparent in both types of control region in both species. Comparison with other sequences deposited in GenBank did not give any information as to the function of this region. Due to this and the very high level of A+T residues (94.4% *C. vicina*; 97.2% *C. vomitoria*) this was designated as part of the control region and thus called A+T rich region 'C'. Even though this region does not appear to have a function, it is still subject to some level of mutational restriction as both species are 71bp long and there were no insertions/deletions in any of the samples sequenced in this study.

This region is in an equivalent position to the *Chrysomya* Intergenic Region (CIR) as nominated in the work of Lessinger *et al.* (2004). The CIR is also 71bp in length and on alignment with the *Calliphora* sequences from this study show approximately 50% identity.

This part of the control region was found to be more useful in population differentiation than region A. The AMOVA for *C. vicina* populations indicated a statistically significant difference between the population genetics of this region. It shows that random mating is not occurring within the species as a whole but within the populations themselves. However, the isolation by distance test using the Mantel correlation coefficient indicated that this population difference in genetics is not related to geographic distance. The tree highlights a definite Mitcham Junction and Wimbledon grouping, although the confidence values after bootstrapping for this branch is low.

For *C. vomitoria*, the AMOVA result was not significant at the 5% level but was 0.133, which is significant at the 10% level. The Mantel correlation coefficient of genetic and geographic distances was however significant (at 5%), and this indicates that there is a relationship between genetic and geographic separation of the *C.vomitoria* populations. Indeed the phylogram indicated several distinct groupings, although most were mixtures of the populations. One grouping did solely consist of Keswick samples. It is probably this defined genetically different group that produced the significant positive correlation coefficient. However, one of the Keswick samples was grouped elsewhere and therefore this region cannot be used to differentiate *C. vomitoria* Keswick population from other UK populations.

9.3.6 Nuclear pseudogenes

A possibility for the presence of the two types of control region evident in this work could be that one is in fact a pseudogene within the nuclear genome. These are also known as numts (NUclear copies of MiTochondrial Sequences). Sorenson and Fleischer (1996) suspect that numts are ubiquitous but are often missed by researchers. These numts may affect the reliability of PCR results. Pseudogenes are not transcribed to produce viable proteins and therefore are able to accumulate mutations. This can lead to variations in size from the original gene. They would show up as multiple bands on a post-amplification gel. Zhang and Hewitt (1996a) showed that the entire control region of the desert locust (*Schistocera gregaria)* is present within the nuclear genome. They demonstrated this by amplification of the control region in separated mitochondrial and nuclear DNA fractions – multiple banding present after amplification of total DNA was not evident when the separated fractions were amplified.

Separated fractions were amplified in this work too. As expected only the nuclear fractions amplified the nuclear gene, actin. However, both fractions amplified the mitochondrial COI gene. There could be two reasons for this, one is that there is actually a COI pseudogene within the nuclear genome and the other is that the nuclear fraction contains some mitochondrial DNA. All negative controls in this experiment were negative and so contamination can be ruled out.

Bensasson *et al.* (2000) indicated that multiple copies of the COI region were present in the grasshopper by the presence of different fragments within an individual after digestion with enzymes. Sunnucks and Hales (1996) also found COI numts in the aphid *Sitobion* genus and whilst the level of variation between the sequences was low, they were not identical. This could explain the appearance of COI amplicons from the nuclear fraction. In Chapter 6, over 200 COI regions were sequenced and none showed any intra-individual variation. While this does not prove that COI numts do not occur, if they are present, they either have identical sequences to their mitochondrial counterparts or are at too low a level to be detected on the Genetic analyser. More likely is that the method of organelle separation did not work that efficiently and some mitochondria were extracted in the nuclear DNA fraction.

As the mitochondrial DNA fraction does not appear to contain any nuclear DNA the results from the control region amplifications can still provide some insight to the location of the different types of control region. The presence of A+T rich region A amplicons within the mitochondrial DNA fraction implies that the type 1 control region is present in mitochondria. Likewise the presence of multiple banding in the mitochondrial fraction when the total control region is amplified indicates that both types of control region are present in the mitochondrial fraction.

Whilst these results can not indicate whether control region numts exist, they do indicate that heteroplasmy of the control region does exist in *C. vicina* and *C.vomitoria* mitochondria. Rand and Harrison (1989) also demonstrated that with pure mtDNA extraction, length heteroplasmy was still present in crickets. The tRNA duplication was observed in *C. chloropyga* by the Lessinger research group in both mitochondrial and total DNA extractions (Lessinger *et al.* 2004, Junqueira *et al.* 2004). They did not observe the presence of two types of control region within the mitochondrial fraction alone and also in the presence of nuclear DNA.

This heteroplasmy could explain why tRNA gene duplication has only been noted in some species of Calliphoridae and not others. It could simply be due to researcher error – if the region is cloned before sequencing, it could be that only clones of one type are being sequenced. Alternatively, the common ancestor could have been heteroplasmic and in some species this heteroplasmy was lost perhaps due to bottleneck events.

One factor not accounted for by these results is the possibility that the heteroplasmy observed is actually due to tissue specific mitochondrial genomes. These were first observed in human disease patients (reviewed in Ballard and Dean 2001) and are inheritable. This study did not separate tissues before DNA extraction and so it is not know whether this would explain the variation observed in mitochondrial control regions.

The presence of numts are detrimental to researchers trying to explore the evolutionary history of a species through maternal lineages as once transposed to the nuclear genome these regions will be subject to both maternal and paternal

inheritance. Zhang and Hewitt (1996b) demonstrate, in their review of the subject with the data of Arctander (1995), the difference using a mtDNA and its nuDNA homologue makes to the eventual maximum parsimony phylogram produced. Different species are grouped together on the separate phylograms.

For the forensic entomologist however, the objective is identification. The presence of numts may even be beneficial as they could potentially increase the variation between populations. As long as entomologists appreciate that these nuclear pseudogenes may be present and that they will not have the same properties as mitochondrial markers (e.g. maternal inheritance, haploidy etc), then these gene regions should still be viable as identification markers.

Chapter 10

Discussion and Conclusions of Part I

10.1 Trap site locations

The methods of trapping in this study all achieved reasonably high levels of blowfly. *Calliphora vicina* has already been established as a synanthropic species compared with *C. vomitoria*, a more rural species, and this was reflected in the flies caught at the variable trap sites. The Cheltenham and Wimbledon populations of *C. vicina* were caught within the town in the vicinity of houses and the *C. vomitoria* on grassland near woodland only 3km away (Wimbledon common and Leckhampton hill, Cheltenham).

Trap site locations outside of the London area were chosen to be of relatively great distance from each other – more than a blowfly might be expected to travel in a short period of time. These populations represent flies that would not arrive at the same carrion and therefore can be thought of as separated.

10.2 Intraspecific differences in developmental rate

One of the reasons to distinguish between populations is the possibility of differences between developmental rates. This was tested between *C. vicina* populations from Cheltenham and Waterloo. The results showed that it was worthwhile trying to distinguish populations as although the ADH to adult was similar, the developmental rate of some immature stages were was significantly different.

This work began with the Cheltenham population of *C. vicina* and it was hoped to extend this side of the research to other *C. vicina* populations and also *C. vomitoria* from around England and Wales. After the first developmental differences between populations were noted it was decided that the main focus of this research should centre on the molecular identification of these blowflies. Once molecular markers have been located, focus should then return to examination of the population developmental rates. As it has been established in this work that statistically significant differences exist between two populations of *C. vicina*, located approximately 140km apart, it would be prudent to examine the developmental

timings of other *C. vicina* populations. The developmental rates of a population further north than Cheltenham and London should be elicited. Even though it was calculated from weather station data that London temperatures were on average 1.4°C higher than Cheltenham for the year 2003, the difference between London and Keswick for the same year is 3.2°C. It is likely that these temperature differences will have altered the growth rates of the blowflies in these regions.

10.3 Molecular differentiation of C. vicina and C. vomitoria species

This work indicated that restriction enzymes provide a simple method for highlighting polymorphisms between samples. This was demonstrated with the *Sfc*I digestion of *C.vicina* and *C. vomitoria* COI sequences and it was after other researchers had noted differences in the patterns of *C. vicina* XDH restriction digests that this region was considered as a potential region for intraspecific variation. The problem with utilising enzymes when compared with examination of the DNA sequences is that a lot of potential variation is missed unless many enzymes are employed.

Using the COI region sequences in this study as an example, there are few enzymes that could differentiate between all Calliphoridae. When the total sequences were compared the differences were great enough to separate Calliphorid species as demonstrated in the neighbour-joining tree (aside from the Hawaiian *L. cuprina* hybrids discussed). This work has therefore highlighted the benefits of using complete sequence analysis opposed to restriction enzymes.

The results of this study indicated that COI provides a suitable marker for distinguishing between English *C. vicina* and *C. vomitoria*. The nuclear XDH region examined in this study also showed interspecific markers for *C. vicina* and *C.vomitoria*. However the higher level of intraspecific variation within the XDH region means that COI is better for species identification. After comparison with the sequences deposited in GenBank and later with work conducted in this laboratory (Martin 2004), this region of the COI gene can also be used to distinguish between other Calliphoridae. This work has been submitted for publication.

After experimentation with the DNA extraction and amplification techniques used in this study on various body parts and immature stages, it is clear that a forensic entomologist could differentiate *C. vicina* from *C. vomitoria* regardless of sample type by use of this COI region.

10.4 Differentiation of C. vicina and C. vomitoria populations

The F-statistics calculated from the polymorphisms in the XDH region and the exact test of Hardy-Weinberg equilibrium indicated that mating is not random throughout the English populations of both *C. vicina* and *C. vomitoria*. Similarly, Hale and Singh (1987) noted that the populations of *D. melanogaster* are highly structured despite the flies' ability to migrate far.

The Mantel correlation coefficients for both the XDH and the A+T rich region C sequenced in this study indicated that there is a positive correlation between the geographic and the genetic distance for *C. vomitoria* populations. *Calliphora vomitoria* is subjected to greater artificial transportation as immatures are used as bait for coarse fishing and so has the potential to be moved a great distance. This appears not to affect the population genetic structure of the populations studied and has not caused gene mixing within English flies. It should be noted however that fewer *C.vomitoria* flies than *C. vicina* were included from outside of London and this might have caused bias to the results.

The neighbour joining trees of both XDH and A+T rich region indicated that barriers like the River Thames in London are not an obstacle to migration between *C. vicina* and *C. vomitoria* populations. Stretches of water were observed not to be a barrier to blowfly movement by MacLeod and Donnelly (1958). Hwang (2004) also noted that gene flow within London population was high and traversed both sides of the River Thames.

This is not the case for English *Lucilia* species. Stevens and Wall (1997) showed using RAPD analysis that whilst intraspecific variation existed in worldwide samples of *L. sericata* and allowed separation of British flies from other European flies, the English flies could not be separated. They compared samples from within Southern England (Stevens and Wall 1995) and although some genetic differences are evident in the RAPD analysis the authors concluded that it was of such a low level that *L. sericata* in southern England formed a freely mixed population.

The ultimate conclusion for this type of work is to locate private alleles for each population, thus allowing definite genetic distinctions between blowfly populations. Although some private alleles were located in both species for the XDH region, none were present in all members of the respective populations. Consequently, neighbour-joining trees and/or population assignment methods appear the best way of determining the original population of an unknown specimen. Wells and Sperling (2001) used phylogenetic analysis to determine the species of unknown samples and also to show that degraded samples would still be included in the tree alongside those of full sequence length.

The problem with Paetkau's population assignment method is that it cannot be used on haploid and therefore mitochondrial markers. It did assign many samples to their correct populations and is definitely a method that should be employed when using nuclear markers. The other benefit of population assignment analysis is that likelihood values are given for every sample and population pairing. This provides a ranking of populations that the sample is similar to and also gives a measure of the degree to which the sample is similar to the known population samples. The ability to provide numerical support for a population assignment would be useful if presenting the results as evidence in court.

10.5 Comparison of mtDNA and nuDNA as sources of population markers

This study included both nuDNA and mtDNA as sources of potential markers. It can be concluded that the mitochondrial protein coding genes show little variation. The COI region contained high interspecific variation but very low intraspecific variation. The srRNA and tRNA sequences showed no variation for the samples contained in this study. Due to the difficulty with sequencing the complete mitochondrial control region, only sections of it were sequenced in multiple samples. For these sections of non-coding DNA there was a lower level of variation than in the nuclear coding XDH gene region.

This was also illustrated in the work of Nardi *et al.* (2003). They sequenced the entire mitochondrial genomes of olive flies (*Bactrocera oleae)* from two locations (Italy and Portugal). The genome was 15,815bp in length and yet only 31 nucleotide substitutions were noted between the two populations. This is a level of 0.2%

variation. They concluded that whilst the two populations could be distinguished from each other, the levels of divergence were extremely low. These were not populations that were close to each other; they were from two separate countries and therefore the level of migration between populations in one generation would be near zero (there is always the chance of artificial transportation). The functional constraints on the mitochondrial genome appear to limit the amount of variation.

Another problem with use of the mitochondrial genome, which does not affect its use in identification but does when using mtDNA markers for maternal lineages, is that paternal leakage of mtDNA has been noted in several species, including *Drosophila* (Kondo *et al.* 1990). The presence of numts, as discussed in Chapter 9, also affects the use of the mitochondrial genome as a source of molecular markers for maternal lineage.

10.6 Further work on identification

10.6.1 Samples, populations and species

This work can be extended further, not only to add to the results by including more samples from the populations and species already studied but also other species of forensically interesting arthropods can be examined. For an entomologist to use the COI region to identify an unknown sample all other Calliphoridae, especially the other UK species of *Calliphora,* would need to be examined. Other populations from Britain should be examined to build up a greater picture of the genetic differentiation of *Calliphora* species.

10.6.2 Dispersal Studies

More work needs to be carried out on the dispersal distances of blowfly. Dispersal studies can be mark and recapture style studies. This is where flies are caught, marked, released and recaptured. The studies can be quite biased as traps are placed subjectively and therefore will often miss maximum dispersal distances. A more novel technique is the use of radar technology attached to individual insects to allow tracking (reviewed in Osborne *et al.* 2002).

How far a blowfly will fly to reach carrion needs to be assessed more accurately. This distance may well be limited to the olfactory range of the adult flies. A forensic entomologist is not interested in the natural aggregations of the blowfly but how far

the blowflies arriving at a corpse will have travelled to reach it. More accurate measures are needed of the *Calliphora* species to justify using population data caught in the manner of this study and for the application of the results of studies such as this to crime scene evidence.

10.6.3 Molecular markers in genes related to temperature

Any differences noted in temperature adaptation of blowfly may be due to variations in related genes. These variations may relate to different populations of blowfly. Hwang (2004) postulated that heat shock proteins (proteins involved in thermal tolerance) should be examined as a possible mechanism for coping with the high temperatures within larval masses. These could be examined for molecular markers or differences in relative expression of the genes coding for these proteins could be assessed.

10.6.4 Sequencing of the whole mitochondrial genome

Roehrdanz (1995) and Yamauchi *et al.* (2004) both describe a method of relatively simple amplification of the entire mitochondrial genome of insects. The whole mitochondrial genome could be characterised for both *Calliphora* species to search for more molecular markers. The complete *Ch. chloropyga* mitochondrial genome has been sequenced by Junqueira *et al.* (2004). However, practically all of the genome is coding DNA and, as demonstrated and discussed in this study, shows little intraspecific variation. It would be more productive to concentrate efforts to find other molecular markers away from the mitochondrial genome.

10.6.5 Nuclear genome

Focus is beginning to switch from mitochondrial COI, which has been heavily used in entomology to the nuclear genome (Harvey *et al.* 2005, Wallman *et al.* 2004).

10.6.5.1 Internal transcribed spacers regions

Within the eukaryotic nuclear genome, the ribosomal DNA genes are arranged in randomly repeated multigene complexes. Each complex consists of 28S, 5.8S and 18S coding regions that are interspersed with internal transcribed spacer regions (ITS). ITS1 is located between 18S and 5.8S and ITS2 is between 5.8S and 28S. These non-coding regions are transcribed along with the genes and are later spliced out. The role of these ITS regions is unclear but due to their high evolutionary divergence rate they

are not thought to have a specific function but are more likely involved in the processing of the ribosomal genes.

Previous studies using these regions have used RFLP. Ratcliffe *et al.* (2003) demonstrated that digestion of ITS1 and ITS2 by the restriction enzyme *Ssp*I produced fragments that separated five Calliphoridae species. ITS1 and ITS2 have also been subjected to DNA sequence comparisons. The conserved ribosomal genes flanking the ITS regions can be used to design universal primers for subsequent PCR applications. Hillis and Dixon (1991) designed a series of universal primers based upon fungal species for these regions. The problem with using primers designed to be universal to all species is that they can amplify up any DNA contamination along with the DNA from the subject. There is also a possibility that they could amplify any gut contaminants i.e. parasites. Also the problem with using primers designed for a completely different phylum is that they may cause mispriming due to sequence differences even between conserved gene regions. Ji *et al.* (2003) have designed primers for this region solely based upon conserved regions within invertebrate/insect data sequences deposited in GenBank.

Parkin and Butlin (2004) discovered when using ITS1 in the meadow grasshopper, that this region contained a very high level of intra individual variation (over 85%), along with a fairly high level of intra population variation (over 6%). Vogler and DeSalle (1994) also discovered this with the ITS1 region with tiger beetles and commented that this complicates the use of ITS1 as a marker in phylogenetic analysis. Schlötterer *et al.* (1994) suggest that the levels of intra individual variation will vary according to species and therefore this region should be examined for *Calliphora* population markers.

10.6.5.2 RAPD Markers

It has been established that the use of random primers can often produce results that are not reproducible from laboratory to laboratory (Black 1993). However, RAPD analysis can be a worthwhile technique if it is taken further than simply analysis of amplicon patterns on gels. Once a band on the gel has been determined as differentiating between populations, it should be excised and the DNA sequences established. Specific primers to this sequence can then be designed, thus removing the need for random primers in later analyses. These specific primers would need to

be tested to demonstrate that they were capable of differentiation and were not from a amplicon that had become evident after some RAPD PCR anomaly.

10.6.5.3 Microsatellites

Microsatellite sequences are short tandem repeats found scattered throughout the genome. The number of repeat units is highly variable and has been shown to vary within species. To locate markers a library of genomic DNA for a particular species needs to be constructed. This is then interrogated with oligonucleotides consisting of repeat sequences. Once located, the repeat sequences are amplified by PCR, using primers that flank the repeats. Amplicons are separated by electrophoresis and the differences in fragment length will indicate different numbers of repeats. Microsatellites have already been used with varying degrees of success in *C. capitata* (Bonizzoni *et al.* 2000) and *Drosophila* species (Frydenberg *et al.* 2002, Schlötterer and Agis 2002). Rodrigues *et al.* (2004) in Brazil have begun work to locate microsatellites in Calliphoridae.

This work then examined potential molecular markers for determining the age of immatures forms of *C. vicina*.

Part II

Identification of temporal patterns of gene expression in forensically important blowflies

Chapter 11 Introduction to temporal gene expression

The ability to predict the development of a species under certain temperature conditions allows a forensic investigator, using recent climatic data from the crime scene, to estimate the minimum PMI.

It is relatively simple to determine what stage a *Calliphora* insect is at morphologically. The larval stages can be distinguished by the number of posterior spiracles. The first instar has one spiracular opening; two slits are present in the second instar and three slits in each spiracle for the third instar. The pupal stage naturally appears distinct from the larval stages.

Less simple is deducing the age of an insect, as discussed in Section 1.4.9 age can be estimated using isomegalen-diagrams (Section 1.2) but these do not apply to the pupae. As demonstrated in Figure 1.1, this is the longest immature stage in the *Calliphora* lifecycle. The pupal stage can be broken down into phases because as the puparium hardens (sclerotises) at the beginning of this stage the external cuticle goes through a series of colour changes. This change begins with white, through shades of orange to dark red (personal observation). This process of tanning of the cuticle occurs over several hours and can therefore be used as an age marker for the first day after white puparium formation. Indeed Greenberg reports upon a case whereby colour photographs of an autopsy showed lightly tanned puparia allowing an estimation of minimum time since death (Greenberg 1991).

Vincent and Ablett (1987) showed a link between the tanning process and *C.vomitoria* cuticle contraction, thus releasing water. The gradual dehydration could also be used as a method of aging pupae – either by overall weight or by measurement of free water present.

As metamorphosis continues, adult structures begin to be formed within the puparium and the eyes go through distinct colour changes (as noted for *P. regina* Greenberg 1991).

To use these age markers pupae need to be stripped of the puparium. Most of the pupal morphological changes have yet to be linked to temperature/time for all forensically important species.

An alternative is to utilise molecular techniques to determine the age of insects. As insects age, a variety of genes are expressed for the organism to develop. Gene expression is often transient in nature, with some genes being switched on/off for only short periods. If this gene expression can be related to specific times during the *Calliphora* lifecycle, the genes would provide molecular markers for determining the age of insects.

11.1 Gene expression

As discussed in Section 3.1 the genome contains the information required to produce proteins. The sequence within the DNA polymer dictates the amino acid sequence synthesised which naturally determines the eventual protein.

Protein synthesis begins with transcription of DNA to RNA and this is initiated by transcription factors. One strand of the DNA molecule is copied into a complementary preliminary messenger RNA molecule (mRNA). The introns are then spliced out of the mRNA molecule and the exons joined together.

Transfer RNA molecules (tRNA), complementary to a particular mRNA codon and linked to the respective amino acid, bind to the RNA molecule thus allowing the amino acid to join the growing amino acid chain, eventually producing a protein. This process is known as translation.

The transcription and subsequent translation of each protein is not a continuous process. Genes are transcribed at specific times as a result of the production and binding of transcription factors and are thus capable of being 'switched' on or off.

Therefore, whilst the genome contains all the genes capable of being expressed by an organism, it is the mRNA population of molecules within the cell that indicate which genes are being expressed at a given time. It is consequently the mRNA that needs to be probed for potential temporal molecular markers.

11.2 Measuring Gene Expression

The level of gene expression within cells can be measured in several ways. A traditional method is Northern blotting. Total cellular RNA is extracted and denatured usually with formaldehyde and separated by gel electrophoresis. Following this the RNA is transferred to a nitrocellulose membrane and exposed to a labelled probe. This is then visualised using autoradiography. The amount of RNA in the sample can be estimated using densitometry.

An alternative method is to reverse transcribe the mRNA back to DNA. This DNA is known as complementary DNA (cDNA) to differentiate it from genomic DNA. It differs from genomic DNA as it is made of coding DNA only (all the introns were spliced out of the preliminary mRNA molecule *in vivo* before RNA extraction and subsequent reverse transcription). Known genes can then be amplified by PCR from this cDNA and the products quantified. This is commonly done using real time PCR, whereby the samples are quantified during the PCR cycling.

A disadvantage of both the above methods is that they require prior knowledge as to which genes to look for. The sequence of these genes is also needed for the production of either primers or probes. A method that has been utilised in recent years for analysing differences in gene expression is microarray technology. It is not necessary to know which genes have varying patterns of expression before experimentation begins as it examines all the genes within a genome simultaneously. A microarray is typically a glass slide upon which tens of thousands of oligonucleotides are attached. These DNA fragments are complementary to known genes within a particular organism.

There are several ways of comparing gene expression of different conditions (e.g. timepoints/disease states) using microarrays. The simplest method is to hybridise fluorescent dye labelled cDNA from one condition to an array and compare the

positions where fluorescence is emitted, after excitation with a laser, with arrays from other conditions. If just two conditions are to be compared (often control versus disease) these can be done on the same array using two different dyes for the different conditions.

While the microarray approach does not require prior knowledge of which genes to look for it does require prior knowledge of the organism's genome for the relevant oligonucleotides to be designed, synthesised and 'spotted' onto the slide. Microarrays have been assembled for frequently used research organisms e.g. human, mouse, rat, and yeast. There is also a *Drosophila* chip made by Affymetrix®. If this could be used with the *Calliphora* species, the entire genome could be scanned for samples at various timepoints to locate molecular markers. However, Affymetrix® do not recommend the use of array chips for alternative species although Kayo *et al.* (2001) have used human GeneChips® with rhesus monkey (*Macaca mulatta*) samples. Humans and rhesus monkeys have >95% similarity between genomes and are thought to have diverged 25 million years ago (Stewart and Disotell 1998). *Drosophila* and *Calliphora* are thought to have diverged between 80-99 million years ago (Rocher-Chambonnet *et al.* 1987; Beverley and Wilson 1984). Also, gene homology between the genera are much lower that that of the human and monkey, for example the XDH gene examined previously has 66% sequence identity between *D. melanogaster* and *C. vicina*.

As suitability of the *Drosophila* GeneChips® for *Calliphora* samples could not be ensured it was decided not begin this work using microarray technology, although the possibility of using them in future analysis should not be ruled out including the production of custom-made *Calliphora* chips capable of identifying stage and species. Instead cDNA made from extracted total mRNA would be probed with primers for specific genes individually in order to get more insight into the *Calliphora* developmental genes.

11.3 Rationale of Part II

The aim of this section of work would be to use molecular techniques to accurately determine the age of a discovered insect. Using *C. vicina* as the sample species, genes expressed at specific time points during the blowfly lifecycle could be identified.

Initially the pupal stage would be focussed upon, as it is the longest immature stage during which it is difficult to determine the insects' age. This work would be later extended to other immature stages.

The following procedures would be undertaken to achieve this. The first is method development including RNA extraction method and exploration of potential molecular markers. Expression of these markers would then be quantified, initially by endpoint quantification after amplification and then by real time PCR.

Chapter 12

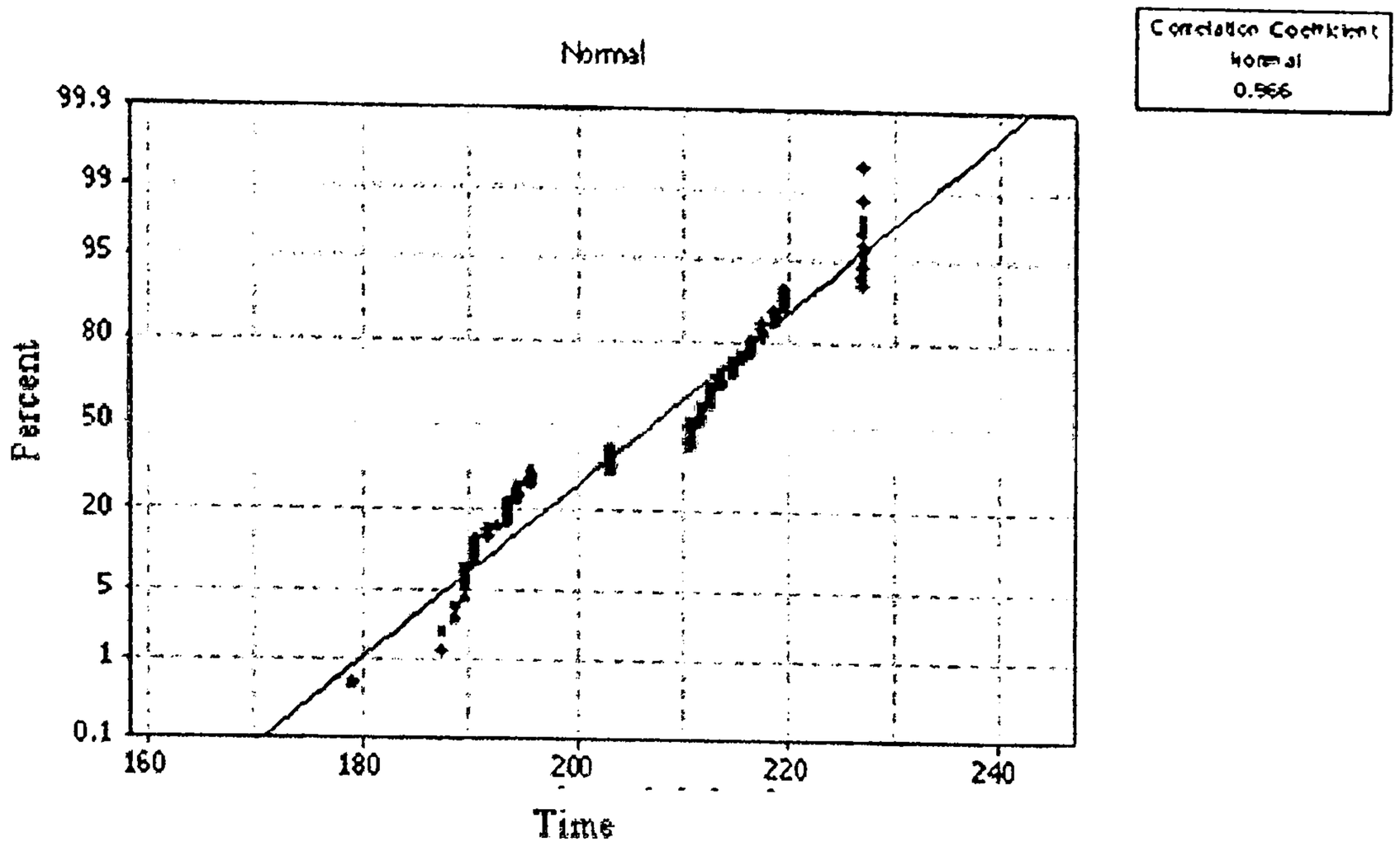
Sample Collection and RNA Extraction Methodologies

The aim of this gene expression research was to compare gene expression at various timepoints throughout the pupal stage of *C. vicina*. As mentioned previously this stage of the lifecycle is approximately 14 days in duration at 20°C (Ames and Turner 2003). The pupal stage would be sampled at regular 24h timepoints. The insects would be kept at 20°C for their entire lifetime corresponding to daily sampling points every 480 ADH.

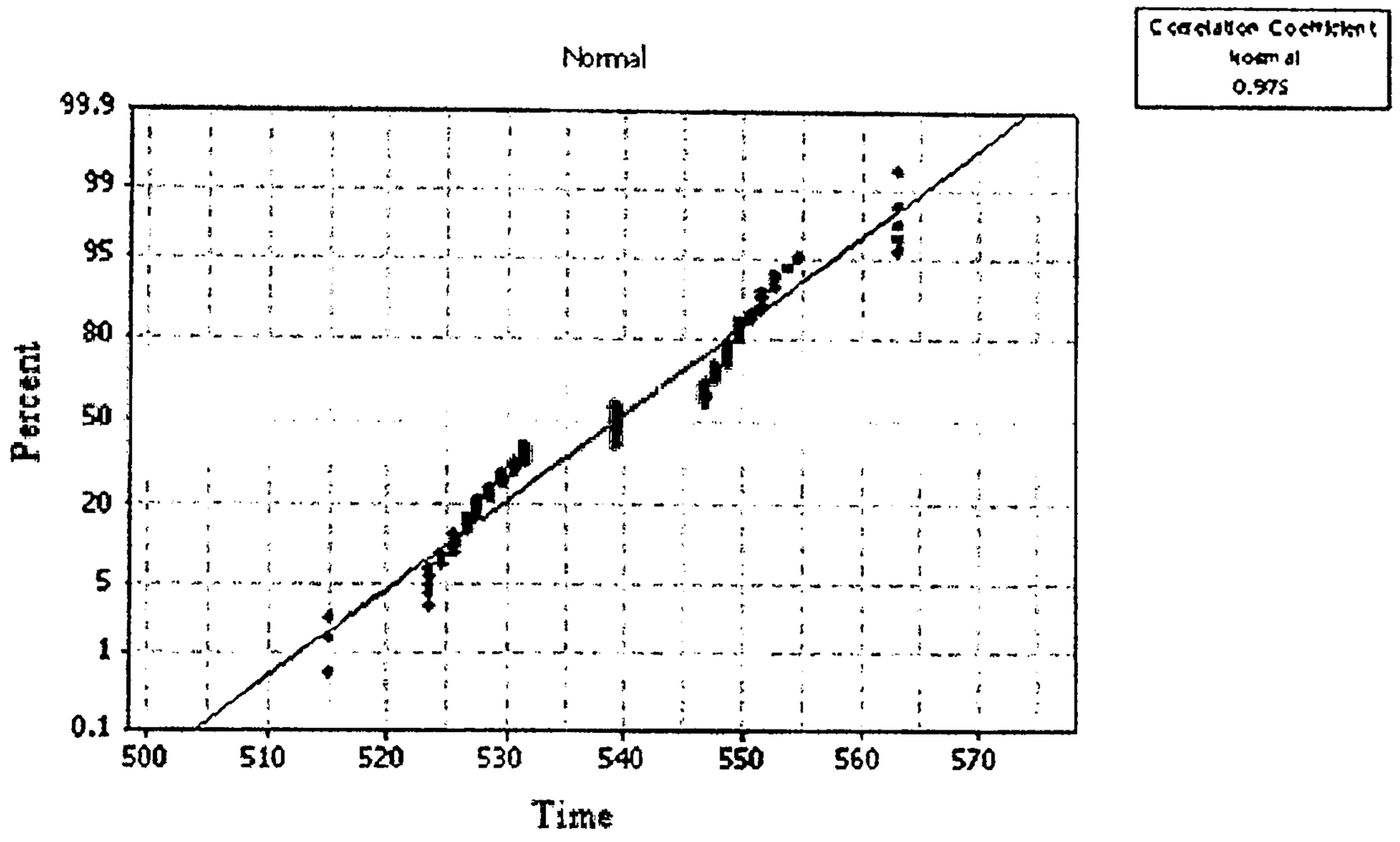
The initial method of sampling pupae was such that an entire egg batch was placed at 20°C and allowed to develop. At 24h time points during the pupal stage a set number of pupae was removed for RNA extraction.

However, personal observations of *C. vicina* developmental timings and the results of lifecycle data in the present work (Chapter 4) indicates that natural variation exists within an egg batch, so that all insects do not develop at exactly the same rate. Indeed when sampling in this manner was attempted, it became clear that many pupae would need to be removed to get a fair representation of pupae for that age group. Sampling could become quite subjective during the early pupal stage, when the colour of the puparium provides some indication of stage of development.

To illustrate this further, Figure 12.1 shows graphs based on the lifecycle data of Cheltenham population of *C. vicina* from Chapter 4. The graphs plot the time (hours) at which the insects morphed into the pupal or adult stage compared to percentage cumulative frequency. The data are correlated to the regression line for the theoretical Normal distribution calculated for this data (based on the mean value and standard deviation). Data were also compared to other distributions including Log Normal and Exponential (data not presented) but the Normal distribution produced the best correlation. MINITAB software was used for this analysis.



A



B

Figure 12.1. Cumulative frequency of number of *C. vicina* (Cheltenham population) reaching the pupal (A) or adult (B) stage over time (hours). Regression line represents the normal distribution and correlation coefficients indicate closeness of fit to this distribution.

There was correlation between the experimental results and the regression line for normal distribution (Correlation coefficients of 0.966 and 0.975 for the pupal and adult stages respectively) indicating that the data are normally distributed. This experimental set-up does not allow for examination of the distribution of the duration pupal stage, as development on an individual basis was not measured. Readings were

taken for a cohort of insects simultaneously. Higley and Haskell (2001) estimated that variation between individuals could be up to 10%.

From the data, the start of the *C. vicina* pupal instar can vary from 179 to 227h at 20°C (this range is equivalent to 2 days) and the emergence of adults can vary from 515 to 563h at 20°C (range is equivalent to 2 days). This difference could bias the experiment if few pupae from an egg batch are sampled every 24h.

To overcome this it was decided to use an entire egg batch for the samples of one timepoint. Each timepoint would therefore encompass the natural developmental variation seen in these insects.

12.1 Sample Egg Collection

Samples were taken from laboratory populations of *C. vicina* kept as in Section 2.3. Oviposition was encouraged by presentation of protein (pig liver) to females. The first eggs in a period of oviposition were discarded to avoid inclusion of precocious larvae as described in Section 1.4.3. Eggs were immediately taken and positioned using soft forceps or a small paintbrush onto a fresh piece of pig liver (15-20g) and placed in a container (see Figure 2.6) at 20°C. This was repeated so that enough containers to cover the pupal instar were obtained. This whole procedure was repeated six months later for the second replicate of the whole experiment. The number of eggs taken for each experimental condition ranged from 7 to 45 in the first experiment and 8 to 30 in the second. Due to limitations in the number of females ovipositing, fifteen treatments were set up in the first complete experimental replicate and sixteen in the second.

When placed at 20°C each sample container was arbitrarily designated as to when during the pupal stage the container would be removed for experimentation. Time of oviposition was taken as 'time zero' and experimental treatment times were worked out from this point.

Experiment 1 contained the following timepoints 2880, 3360, 3840, 4320, 4800, 5760, 6240, 6720, 7200, 7680, 8640, 9120, 9600, 10080 and 10560 ADH.

Experiment 2 contained the following timepoints 3360, 3840, 4320, 4800, 5280, 5760, 6240, 6720, 7200, 7680, 8160, 8640, 9120, 9600, 10080 and 11040 ADH.

12.2 Sample Pupal Collection

At the appropriate timepoints containers were removed. The pupae within were counted and external colour noted (Appendix IX).

12.3 Pre-extraction preparation

When extracting and working with RNA it is imperative to maintain a clean, sterile ribonuclease free environment. To remove RNases from disposable plasticware, 1.5ml tubes and micropestles (Eppendorf) were soaked in 0.1% diethyl pyrocarbonate (DEPC, Sigma, UK) overnight at room temperature. Containers were then autoclaved, allowed to cool, the liquid removed and the plasticware air dried.

Separate pipettes were kept for RNA work only and radiated regularly using a UV Crosslinker (UVP Ltd, Cambridge, UK). Surfaces were cleaned with DNAzol and Microsol (Anachem), which remove bacteria and nucleases.

12.4 RNA Extraction

Different methods of RNA extraction were experimented with including Midas Invertebrate RNA extraction spin column kit (BioGene, UK) and TRI reagent (Sigma, UK) before TRIzol reagent (Invitrogen, UK) was discovered to be the best method for pupal RNA extraction. This protocol is based upon the guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987) and is adapted from the TRIzol manufacturer's instructions. Samples were placed in liquid nitrogen to flash freeze and immediately placed individually into 1.5ml DEPC-treated Eppendorf tubes containing 1ml TRIzol reagent (Invitrogen, UK). Samples were then homogenised using DEPC-treated micropestles (Eppendorf, UK). The tubes were vortexed briefly before being allowed to stand at room temperature. After 5 min, samples were centrifuged for 10 min at 4°C, (12,000 x g). The supernatants were then transferred to centrifuged Phase Lock Gel tubes™ (Eppendorf, UK). Chloroform (200µl) was added to each tube, shaken for 15s and left at room temperature for 15 min. Tubes were then centrifuged for 15 min at 4°C (12,000 x g). After centrifugation the phase lock gel in the tubes lies between the aqueous (containing the RNA) and organic phases. This allows efficient removal of the aqueous phase without interference from the organic layer. The aqueous phase was transferred to clean 1.5ml DEPC-treated tubes and 50µl of chilled 100% isopropanol was added. Tubes were inverted once to mix and left at room temperature for 5 min. Samples were

centrifuged for 10 min at 4°C (12,000 x g). This separates the RNA from the DNA pellet. The supernatants were transferred to new 1.5ml DEPC-treated tubes. Chilled isopropanol (450µl; 100%) was added to each tube, mixed by inversion and left at room temperature for 10 min. Samples were then centrifuged for 10 min at 4°C (12,000 x g). The supernatant from each tube was removed and discarded. The remaining RNA pellets were washed by adding 1ml of 75% ethanol (diluted with DEPC-treated water), vortexed and centrifuged (7,500 x g) for 5 min at 4°C. RNA pellets were dried briefly under vacuum and 100µl of DEPC-treated H_2O (previously heated to 55°C) was added to each. Each sample was placed at 55°C until the pellet had dissolved and then placed on ice.

12.5 DNA Removal

Any remaining DNA within the RNA samples was removed using DNA-*free* ™, a DNase kit from Ambion (UK) according to manufacturer's instructions. Residual DNA in samples would interfere with subsequent cDNA gene region amplifications. Buffer (10µl of 10X) was added to each of the 100µl RNA samples. Next, 1µl of DNase I was added and each sample was mixed gently. Samples were then placed at 37°C for 30 min, then 1µl of DNase I was added and samples were incubated for 30 min at 37°C. DNase inactivation agent (1µl) was added to each sample before they were mixed gently and left at room temperature for 2 min. Samples were then centrifuged at 10,000 x g for 5 min at room temperature. The supernatants were then transferred to fresh 1.5ml tubes.

12.6 Quantification of RNA

The concentration of the RNA extracts was established by UV spectroscopy on the (WPA UV1101 Biotech Photometer) using UVettes (Eppendorf). Absorbance was read at 260nm and the following formula applied to the results.

$$\text{Concentration of RNA (µg/ml)} = \text{Absorbance}_{260} \times \text{dilution factor} \times 40$$

Quantification results for all RNA samples can be located in Appendix IX.

12.6.1 Preliminary Extractions

Before experimentation began on the pupal samples described above, the RNA quality using this extraction protocol was assessed on six *C. vicina* pupae. After RNA

extraction and quantification using UV spectroscopy (Table 12.1), the samples were run on an Agilent Bioanalyser 2100. The Agilent Bioanalyser uses capillary electrophoresis technology. Samples are loaded onto chips specific for RNA and fragments are separated according to size. Samples are sized by the inclusion of a fragment ladder of known sizes. All wells on the chip also contain internal size standards to account for well-to-well variation. Raw data is presented as an electropherogram. The Agilent Bioanalyser software converts this to a conventional gel view (Figure 12.2). The height of the peak (determined by level of fluorescence) is proportional to the intensity of the gel band.

Table 12.1. RNA extractions from *C. vicina* pupae using the TRIzol protocol. Absorbance at 260nm and 280nm were measured by UV spectroscopy. RNA concentrations were calculated using the product of the A_{260} values and the dilution factors, according to the equation in the text.

Sample	Dilution Factor	A_{260}	A_{280}	Ratio A_{260}/A_{280}	RNA Concentration (ng/μl)
1	50	0.512	0.247	2.073	1024
2	50	0.386	0.168	2.298	772
3	50	0.200	0.097	2.062	800
4	50	0.215	0.110	1.955	215
5	50	0.245	0.111	2.207	490
6	50	0.118	0.058	2.034	944

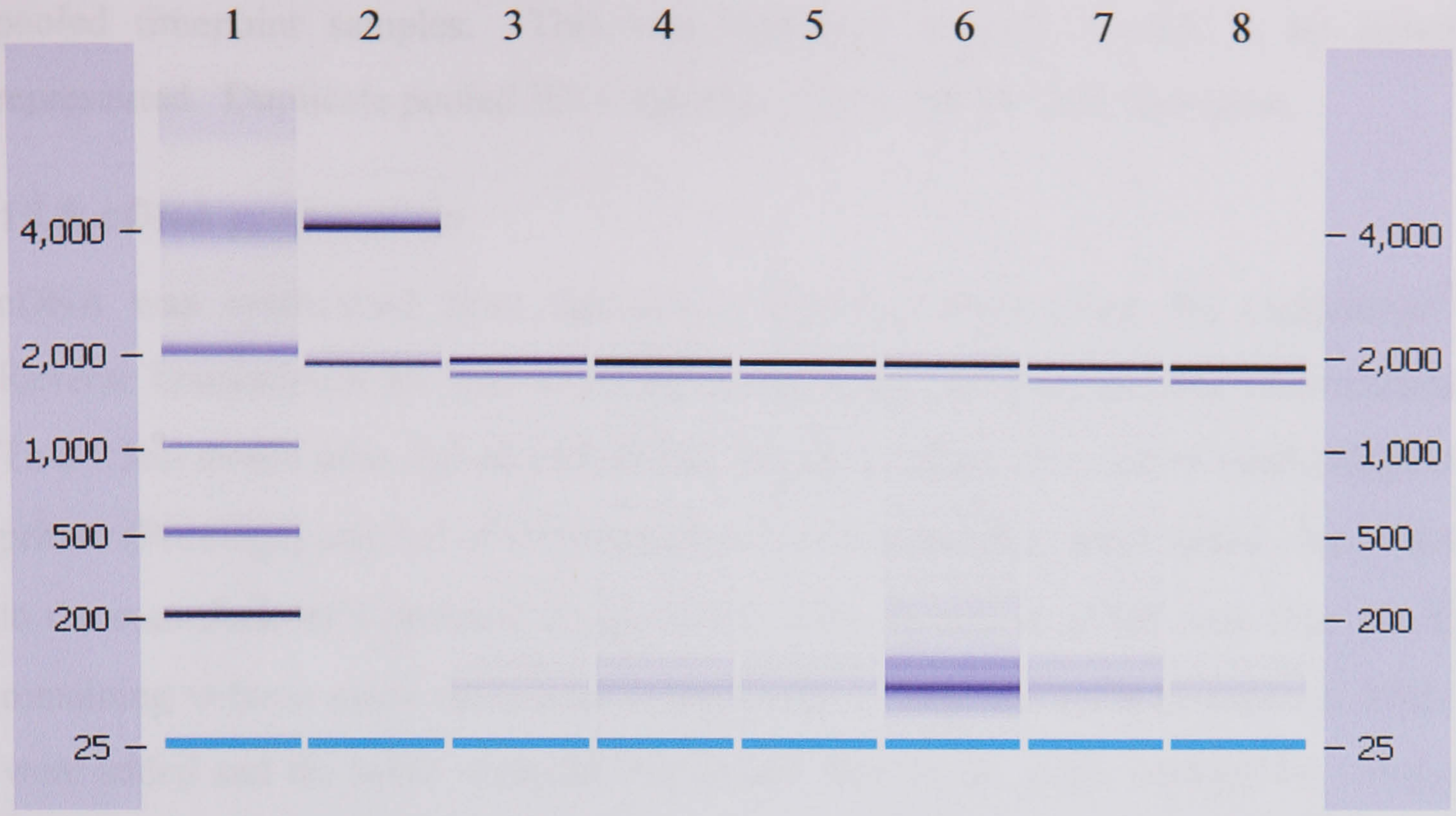


Figure 12.2. 'Gel-like' view produced from electropherograms of *C. vicina* pupal RNA samples. RNA was extracted using TRIzol reagent. Lane 1 contains RNA ladder. Lane 2 contains a human RNA sample (provided by N. Quaderi). Lanes 3-8 contain *C. vicina* pupal RNA samples.

The gel picture indicates the TRIzol protocol is efficient at extracting RNA from *Calliphora* pupae. The fourth sample (Lane 6 in Figure 12.2) shows some degradation, as evidenced by the more intense smaller RNA fragments in the gel picture and the A_{260}/A_{280} ratio <2 in Table 12.1.

For comparison purposes a mammalian sample was run alongside the *C. vicina* pupal samples (results donated by N. Quaderi). This shows the difference in size between 28S and 18S between to the two types of sample. For human ribosomal RNA there is an approximate 2kb difference between 28S and 18S whereas *Calliphora* 28S and 18S rRNA is much closer in size. This concurs with the values found by Whiting *et al.* (2003) on examination of stick insects.

The protocol discussed in 12.3-12.6 was used for RNA extraction on the experimental pupal samples from different timepoints.

12.7 Pooling of RNA Samples

After the individual pupal RNA samples had been quantified they were then pooled within each timepoint. Each pooled timepoint RNA sample contained 1μg of RNA from each individual. Thus each sample contributed the same amount of RNA to the

pooled timepoint samples. This was important as each needed to be equally represented. Duplicate pooled RNA samples were made for each timepoint.

12.8 cDNA preparation

cDNA was synthesised from the mixed extracted RNA using the Omniscript™ Reverse Transcriptase kit from QIAGEN according to the manufacturer's instructions. To a 1.5ml sterile tube, 2µl of 10X buffer, 2µl of 5mM dNTPs, 2µl of 10µM oligo dT primer (Promega) and 1µl of Omniscript reverse transcriptase were added. According to the manufacturer's protocol, 2 µg of total RNA should be added each tube and the remaining volume made up to 20µl using ddH_2O. The calculated volumes of ddH_2O were added and the tubes vortexed briefly and kept on ice while appropriate volumes of RNA extract were added. Samples were next vortexed, centrifuged briefly to spin down the reagents and then incubated for 60 min at 37°C. Remaining RNA was placed at -70°C. After the 60 min incubation the synthesised cDNA was diluted by the addition of 20µl ddH_2O. This was then quantified and then stored at -20°C until required.

12.9 cDNA Quantification

Samples were then quantified on the NanoDrop® ND-1000 spectrophotometer. This spectrophotometer eliminates the need for dilutions in cuvettes as 1µl of sample is applied directly to the measurement surface.

Absorbance readings were taken at 260nm and concentrations calculated according to the following formula.

$$\text{Conc. of first strand cDNA (ng/ml)} = \text{Absorbance}_{260} \times \text{dilution factor} \times 33$$

The cDNA samples were now ready for PCR amplification. Before this could be carried out primers had to be designed. Potential molecular markers referenced in the literature were assessed and also markers obtained by using differential display techniques as discussed in the following sections.

Chapter 13

Design of Primers - Housekeeping Genes

When comparing the gene expression between experimental conditions it is necessary to control for error between samples. This error can be present due to differing efficiencies of the various components of the experimental process. To account for this the expression of a 'housekeeping' or normalisation gene is also measured along with the target gene(s). These housekeeping genes are expressed in all cells and exhibit minimal variation throughout development and under varying experimental conditions. There are various genes that have been previously utilised experimentally as housekeeping genes. These include actins, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), tubulins, 18S and 28S rRNA. They have essential functions within all cells, either as part of the cytoskeletal structure (cytoplasmic actins, tubulins), enzymes in vital biochemical pathways (GAPDH) or ribosomal subunits, hence they are known as housekeeping genes. These genes provide a way of accurate comparison between conditions as normalisation to these housekeeping genes eliminates the discrepancies in gene quantification that occur due to experimental differences. They therefore act as an internal control for comparison between samples. All gene quantifications can be normalised with the amount of housekeeping gene present, and ratios can be compared between experimental conditions.
The genes are also highly conserved and thus allow primers designed for other species to be utilised in amplification.

Actin was chosen as the housekeeping gene for this study. This gene has been used successfully by Canning *et al.* (1996) as a normalisation gene in real time PCR experiments on aphids.

13.1 Materials and Methods

13.1.1 Pupal Samples, RNA Extraction and cDNA Synthesis

Samples were those whose collection, RNA extraction and cDNA synthesis is described in Chapter 12.

13.1.2 Actin PCR

Primers for this gene region were as reported in Canning *et al.* (1996) and are indicated in Table 13.1. Canning *et al.* utilised these to amplify actin in aphid species. They were designed after comparison of conserved regions of a series of invertebrate actin sequences (including *A. gambiae, B. mori, Caenorhabditis elegans* and *Manduca sexta* Linnaeus). The authors note that these conserved primers could be used in variety of invertebrates and when used on *B. mori* produce an amplicon of 320bp.

Table 13.1. Forward and Reverse primers for partial actin gene region from Canning *et al.* (1996).

Primer Name	Sequence of Primer
Actin Forward	5' TTCACCACCACCGCTGAG 3'
Actin Reverse	5' ATACCGGGGTACATGGTG 3'

To check for any residual genomic DNA, RNA extracts were also included in the PCR as negative controls.

Approximately, 50ng of cDNA was added to each PCR tube. Each reaction also contained 0.5µl of both primers (10µM) and was made up to 12.5µl with the appropriate volume of ddH_2O. Readymix™ RedTaq™ PCR Reaction Mix (12.5µl; Sigma, UK) was added after 1 min at 94°C. The following cycles were run ~ 94°C for 3 min; 35 cycles of [94°C for 45s; 59°C for 1 min; 72°C for 1 min]; 72°C for 7 min; 4°C to finish. The thermal cycle parameters are after Canning *et al.* (1996).

Products were visualised on a 1.5% TAE agarose gel stained with ethidium bromide and viewed under UV light.

13.1.3 PCR Product Purification

PCR products were purified using the GFX PCR and Gel Band Kit (Amersham Biosciences, UK) as in Section 5.5. Products were eluted in 30µl ddH_2O.

13.1.4 Sequencing

To ensure that the amplifications were of the correct gene region, two cDNA samples were sequenced in the 310 Genetic Analyser (Applied Biosystems) as in Section 5.6.

These sequences were aligned using BioEdit software. The consensus alignment was compared to those in GenBank using BLAST software.

13.2 Results

Figure 13.1 illustrates an example of actin amplicons produced. The RNA and cDNA for all sample timepoints were amplified and the first seven samples are shown here.

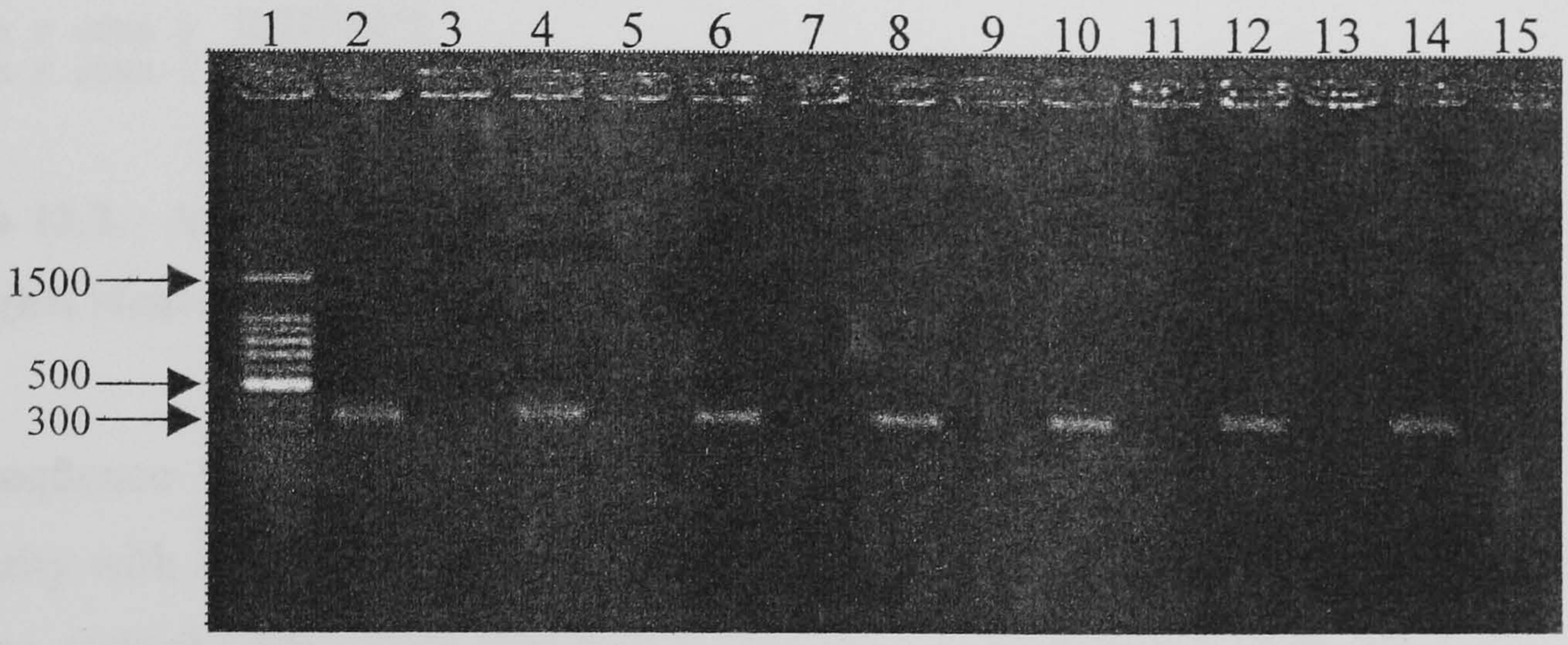


Figure 13.1. Partial *C. vicina* actin amplicons. Lane 1 contains 100bp DNA ladder. The remaining lanes are alternating cDNA and RNA pupal samples.

The amplicons are between 300 and 400bp, which as is as expected after Canning *et al.* (1996), where the *B. mori* amplicon for this region was 320bp. All the cDNA samples showed amplicons. The RNA samples were all negative, indicating that the PCR products from the cDNA samples were due to amplified cDNA and not residual DNA in the samples.

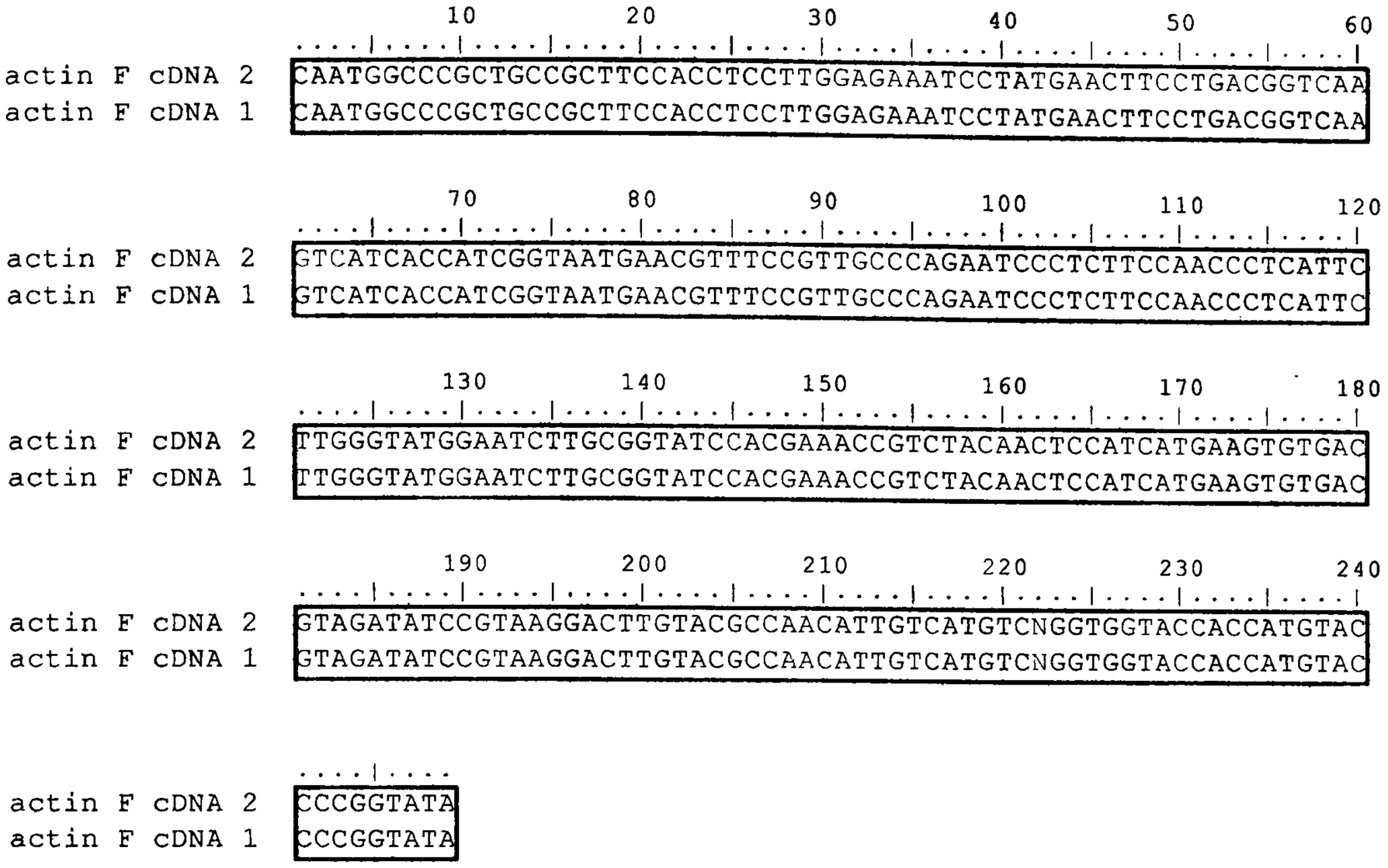


Figure 13.2. Alignment of amplified partial *C. vicina* actin sequences. Black outline highlights identical nucleotides in both sequences.

The sequence was deposited into GenBank (accession number AY954916). The similarity with other insect actin sequences was examined by BLAST software. There were no Calliphoridae actin sequences deposited in GenBank before this study. The closest match is the actin sequence for *Bactrocera dorsalis* (Hendel oriental fruit fly), accession number L12254, which has 217/230 nucleotide matches, expect value $3e^{-95}$. The *B. mori* sequence on which the primers were designed upon, X05185, has 210/241 nucleotide matches with an expect value of $8e^{-59}$.

These primers amplify a *C. vicina* partial actin region in the cDNA samples produced in this study and will be used to measure the gene expression of the actin housekeeping gene later in this work.

Chapter 14

Design of Primers - Potential Temporal Molecular Markers

To identify possible temporal molecular markers for the pupal stage, knowledge of the development occurring within the puparium during this time is needed. Some of the structural changes have been discussed previously but changes on a molecular level need to also be considered. Bluebottles (both *C. vicina* and *C. vomitoria*) have been used by developmental biologists as an alternative invertebrate experimental model to *Drosophila*. Consequently, whilst the amount of knowledge about the genomics of these species is not as great as that of *Drosophila*, there is some information about some of the processes both on the structural and molecular level during the pupal phase of *Calliphora* species.

During the pupal phase, blowflies do not feed and yet they are undergoing heavily energy dependent periods of activity during metamorphosis. The metabolic rate within the puparium is high at the beginning and end of the stage, with periods of protein synthesis corresponding to this high metabolism (Houlihan 1976). Whilst there is little protein synthesis in the middle of the pupal phase there is active growth of adult structures. Houlihan (1976) demonstrated using injections of cycloheximide (which inhibits protein synthesis) that growth of adult flight muscles could still continue, which implies that for some structural changes *de novo* synthesis of protein is not required. These insects must have another mechanism of energy production to develop during the pupal phase.

14.1 Storage Proteins

In holometabolous insects the amino acids and energy for metamorphosis comes from stored proteins, lipids and carbohydrates. They therefore rely on the nutrients that are taken in during the feeding stages of the larval phases. Once absorbed these are then converted into storage compounds. The fat body of insects is the organ responsible for production of these compounds. It corresponds, in part, to the liver of vertebrates (Hansen *et al.* 2002). The fat body of *C. vicina* synthesizes large amounts of protein during the first period of the third larval stage. The majority of these proteins are the

hexamerins, so called because they are made up of six subunits. These proteins are secreted into the haemolymph where they account for approximately 80% of the soluble proteins (Sekeris *et al.* 1977; Burmester and Scheller 1995). At the end of larval development the fat body reabsorbs these proteins to act as a source of amino acids for metamorphosis during the pupal phase. Levenbrook and Bauer (1984) injected labelled hexamerin into larvae and noted which of the adult structures the labelled protein appeared in. They found that the synthesis of most flight muscle proteins involves the incorporation of one amino acid derived from labelled larval hexamerin.

The group of hexamerins are also known as storage proteins, larval serum proteins (LSPs) or arylphorins. There are two main hexamerins that have been identified in holometabolous insects. There are differences in the nomenclature between species in the literature as these proteins were identified and named before their homology between insects was realised. The two main proteins are called LSP-1 and LSP-2 in *Drosophila*, whereas in *Calliphora* LSP-1 is also known as arylphorin or calliphorin (Burmester and Scheller 1992). For this work the term calliphorin will be adopted as it has greater usage in the literature, though it is noted that it is homologous to LSP-1 of other genera.

14.1.1 Calliphorin

The calliphorin gene of *C. vicina* has been sequenced by Naumann and Scheller (1991) and can be found in GenBank with accession number M76480.

This protein has already been examined for tissue specific expression and also stage specific production. Levenbrook and Bauer (1980) found that it was not synthesised before the third larval stage (or that it is not above the level of detection 0.5 x 10^{-6} µg/individual). Schenkel and Scheller (1986) found that RNA from 1st and 2nd larval stage fat bodies gave a very weak signal when hybridised to a calliphorin probe.

Levenbrook and Bauer (1980) detected calliphorin within 1-2 hours after the second moult (all their work was conducted at 25° C), and levels increase rapidly during the third larval stage. According to these authors, the maximum titre, 2.8mg/insect, is acquired between the sixth and seventh day, calliphorin synthesis accounting for two thirds of the increase in total protein within the insect. This level of calliphorin then

drops slightly and remains constant during the pupal instar before declining rapidly two days before adult emergence. Two days post emergence there are only traces of calliphorin detectable in the two day old adult.

Of course the level of calliphorin detected by Western blotting does not indicate that the calliphorin gene is being transcribed and translated. Schenkel and Scheller (1986) carried out a series of Northern blots to detect the presence of calliphorin mRNA. They found that even though calliphorin in the haemolymph was present in the pupal phase, it was not actively being synthesised during this period. Marzini and Scheller (1988) also noted that there was no transcription of calliphorin in white puparia, but that this result might have been obtained due to the limitations of the method. They used Northern blotting to look for the presence of calliphorin mRNA in the total RNA at various stages. They also synthesised mRNA *in vitro* from various stages (with [^{32}P] GTP) and used this to probe a nitrocellulose filter that had calliphorin DNA fragments bound to it. They note that there is a finite amount of RNA that can be applied and thus this method may not be sensitive enough. Schenkel and Scheller (1986) however found that calliphorin mRNA was present in low concentrations in puparia and young adults as well as older adult males and females. This variation in results could be the differences between the sensitivities of different researchers' methodologies.

Levenbrook and Bauer (1980) noted that the calliphorin store is actively used (converted from soluble storage protein to insoluble protein structure) during metamorphosis late in pharate adult development.

Calliphorin is also thought to be involved in sclerotinisation of the cuticle as observed by labelled phenylalanine being incorporated into both the puparium and adult cuticle (König *et al.* 1986; Naumann and Scheller 1991).

Calliphorin has been detected in brain, imaginal disks, salivary glands and epidermis (Schenkel and Scheller 1986) and in haemocytes (Armbruster *et al.* 1989).

14.1.2 Larval Serum Protein-2

Burmester *et al.* (1998) have sequenced the other important storage protein, *C. vicina* larval serum protein (LSP-2). The sequence can be located in GenBank with accession number U89789 and comprises of 2215 bp with a deduced protein structure containing 701 amino acids. This protein is less abundant than calliphorin. Similar to calliphorin, LSP-2 has been examined by Western and Northern blotting to look at its

age and tissue specificities. Burmester *et al.* (1998) found the protein was present in the haemolymph of the third larval stage and in the pupal stage but was not present in any other stages including adults. They located the protein in fat bodies, haemolymph, muscles, gut, epidermis, tracheae, imaginal discs and brains. Northern blotting however only localised the LSP-2 mRNA to the fat bodies, indicating that this is the site of synthesis. The researchers did not find any LSP-2 mRNA present in any stage other than the third larval stage. LSP-2 appears to therefore parallel the expression of calliphorin. It has a very different genomic organisation however; a single gene codes for LSP-2 whereas calliphorin is coded by a gene family of about 20 (Schenkel *et al.* 1985). Burmester *et al.* (1998) comment that although LSP-2 is found to be reexpressed during the adult stages in *D. melanogaster* and *C. capitata* they found this not to be the case for *C. vicina*. Tsakas *et al.* (1991) noted that due to the two periods of LSP-2 biosynthesis in *C. capitata*, the protein was found at a high level throughout the whole of the pupal stage.

14.1.3 Arylphorin Receptor

Burmester and Scheller (1992) postulated that the uptake of arylphorin back into the fat body from the haemolymph was due to receptor-mediated endocytosis. These authors later went on to identify this receptor (Burmester and Scheller 1995) and confirm the complete cDNA sequence for this protein along with its structure. This receptor appears to be expressed from larval day 4 (*C. vicina* were kept at 23° C) until pupation. mRNA was not found in day 3 larvae or in adults. Burmester *et al.* (1998) later showed that this receptor also interacts with the other larval serum protein, LSP-2.

14.1.4 Anterior Fat Body Protein

There is another protein that also binds to the arylphorin receptor but it is not a hexamerin. This is the anterior fat body protein (AFP). The larval fat body of *C.vicina* dissociates into the anterior and posterior parts during metamorphosis (Hansen *et al.* 2002). These authors also report the presence of a protein in the anterior fat body that interacts with the arylphorin receptor. This protein has recently been isolated and characterised in *C. vicina* by Hansen *et al.* (2002). It has been shown in all stages of *C. vicina*, although weakly in the adult stage, and not only in the anterior fat body but also haemocytes.

14.2 Primer Design

Primers for genes of the above proteins were either designed using GenBank sequences inputted into Primer 3 software or were taken from the literature. Table 14.1 lists the sequences of primers used in this work along with their source. It was decided not to include the AFP as other arylphorin receptor proteins would be experimented with initially. If any of these failed then AFP would then be included.

The primer pairs were tested initially on *C. vicina* extracted genomic DNA. Each PCR reaction was set up as in Section 5.4. Amplification was conducted on the 9700 (Perkin Elmer) for the following thermal cycle ~ 94° C for 2 min; [94°C for 30s, appropriate annealing temperature (see sections below) 30s, 72°C for 90s] for 30 cycles; 72°C for 7 min; 4°C to finish.

Amplicons were separated on a TAE agarose gel (1.5%), stained with ethidium bromide and viewed under UV light. If relevant, PCR products were then sequenced as in Section 5.6.

Table 14.1. Sequences of primers used to amplify potential temporal molecular markers in *C. vicina* pupae. Included is the source of the primers or the original template sequence the primers were designed from.

Primer Name	Sequence of Primer	Designed from	Species
Calliphorin Forward	5' CTTCACCGAGGATGTTGGTT 3'	GenBank M76479	*C. vicina*
Calliphorin Reverse	5' TTTGCCAAAGCTTTCGACTT 3'	GenBank M76479	*C. vicina*
Arylphorin Receptor Forward	5' GGTGTTATAATGGATCGAGGCGGACGAGT 3'	Burmester and Scheller (1995) 'R1'	*C. vicina*
Arylphorin Receptor Reverse	5' TCCAGTGTCCATATGAGCAATCTCATCAAT 3'	Burmester and Scheller (1995) 'R6'	*C. vicina*
LSP-2 Forward	5' GGACCTGTCGGTCATGAGTT 3'	GenBank U89789	*C. vicina*
LSP-2 Reverse	5' AGTTGACCGAATCAGGGTTG 3'	GenBank U89789	*C. vicina*

14.2.1 Calliphorin

This set of primers was designed using Primer 3 software on GenBank sequence accession number M76479. The theoretical amplicon size was 301 basepairs. According to the primer manufacturer (Invitrogen) the annealing temperature of these primers is 43° and 47°C (forward and reverse respectively). A thermal gradient PCR was therefore conducted between 40-55°C to establish the annealing temperature for this primer pair.

Figure 14.1 illustrates the PCR products obtained from the thermal gradient PCR.

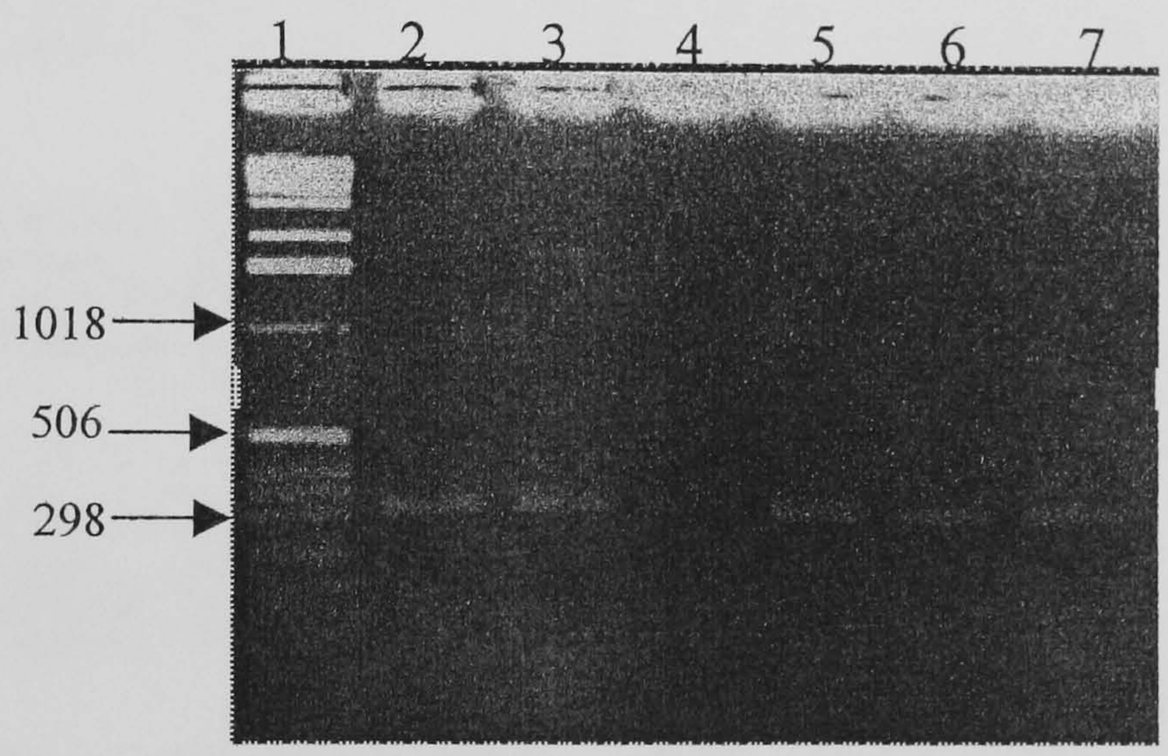


Figure 14.1. Calliphorin amplicons from a thermal gradient PCR. Lane 1 contains a 1KB ladder. Lanes 2 to 7 contain calliphorin amplicons with annealing PCR temperatures of 40, 41.3, 44.2, 48.9, 52.7, 55°C respectively.

From the thermal gradient PCR an annealing temperature of 55°C was chosen, as this is the highest temperature on in the gradient that produced a clear single amplicon band.

These products were then sequenced and the sequence was compared to the M76479 sequence along with other *C. vicina* calliphorin GenBank sequences to illustrate the natural polymorphisms within this gene region (Figure 14.2).

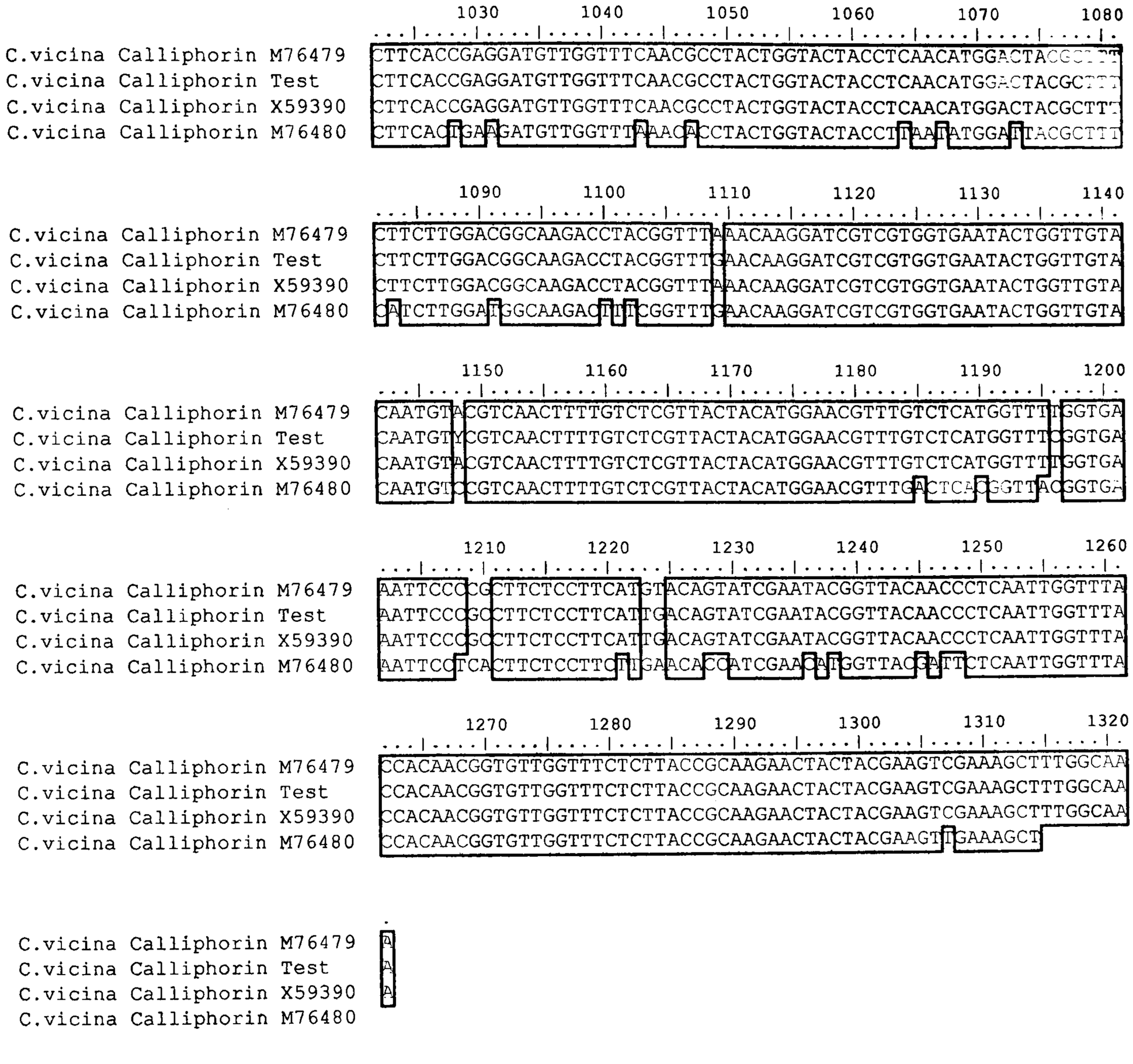


Figure 14.2. Alignment of *C. vicina* calliphorin sequences. The sequence established in this work is labelled 'Test'. Numbering of bases is relative to the complete M76479 sequence. The black outline surrounds identical nucleotides between sequences.

The sequence obtained using this primer pair showed 97.7% homology to the sequence that was used as the template sequence (M76479). The 'Test' sequence had 99.0% and 91.0% homology to sequences X59390 and M76480 respectively. Comparison of these sequences indicates there appears to be natural variation within this gene region.

After using the BLAST facility on the NCBI website (as described in Section 5.7) the calliphorin 'test' sequence had similarities to the arylphorin sequence of *S.crassipalpis* (AF287473; 250/293bp; Expect value $7e^{-62}$), *Musca domestica* Geer larval hexamerin (AY146631, 170/203bp; Expect value $4e^{-27}$) and *D. melanogaster* LSP-1 mRNA (NM_057276; 69/80bp; Expect value $8e^{-10}$). Interestingly the search also yielded a *C.vicina* LSP-2 sequence (CVU89789; 24/25bp; Expect value 0.72).

After noting the discrepancies in the literature as to whether calliphorin was actually being synthesised all through the immature stages, the calliphorin gene region was amplified using cDNA from various aged pupal samples as the PCR template. This was to assess whether calliphorin mRNA was present in the pupal stage. If a negative result in all cDNA samples were obtained the calliphorin work would cease.

A PCR was conducted as in Section 14.2 using an annealing temperature of 55°C and actin and calliphorin primers (Table 13.1 14.1). The samples ranged from beginning to end of the pupal stage in *C. vicina*. Negative controls were included for each sample. These used extracted RNA as the template for PCR to ensure that any positive results were not due to residual genomic DNA left in the samples. Actin was included for comparison. Amplicons were separated on an agarose gel for visualisation (Figure 14.3 abc).

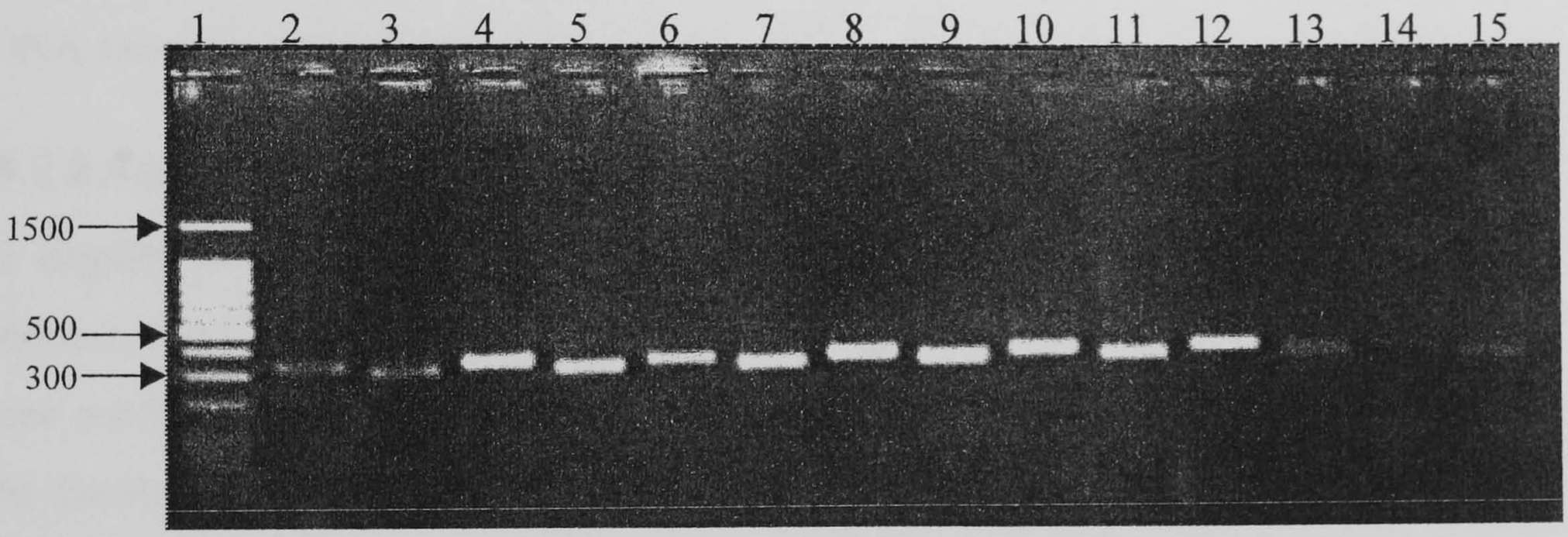


Figure 14.3a. Actin and calliphorin amplicons of *C. vicina* pupae. Lane 1 contains 100bp DNA ladder. Lanes 2-15 alternating actin and calliphorin PCR products early to mid aged pupal cDNA samples.

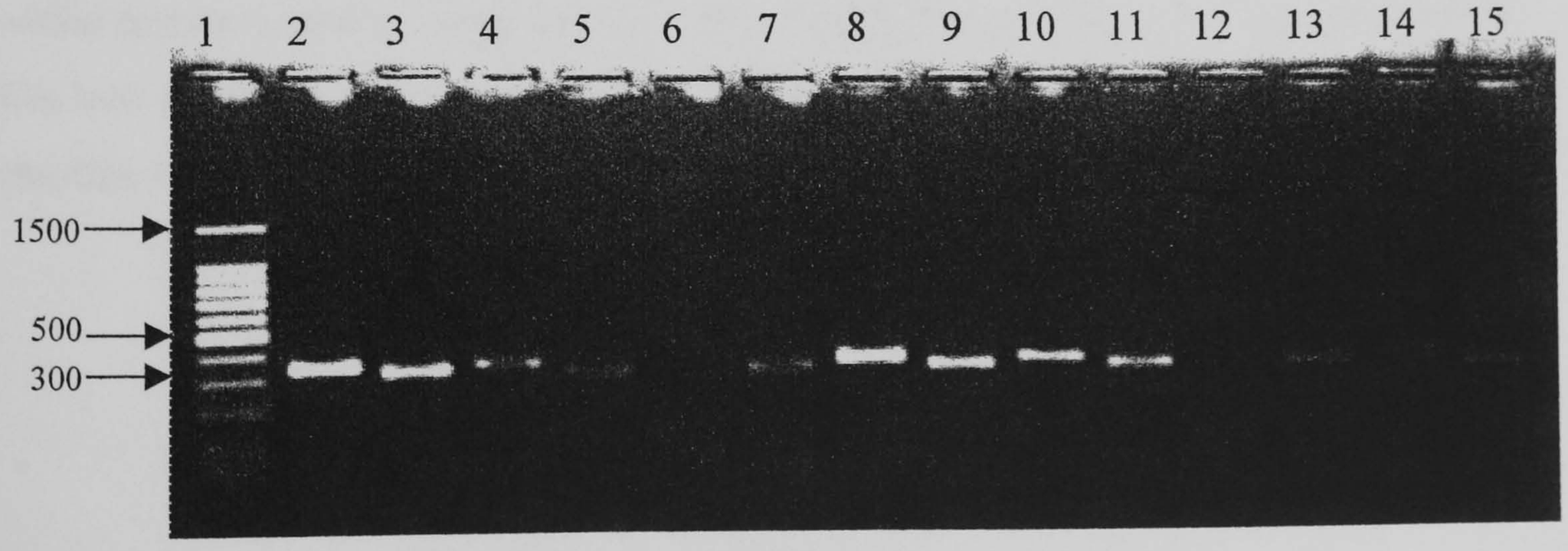


Figure 14.3b. Actin and calliphorin amplicons of *C. vicina* pupae. Lane 1 contains 100bp DNA ladder. Lanes 2-15 alternating actin and calliphorin PCR products mid to late aged pupal samples.

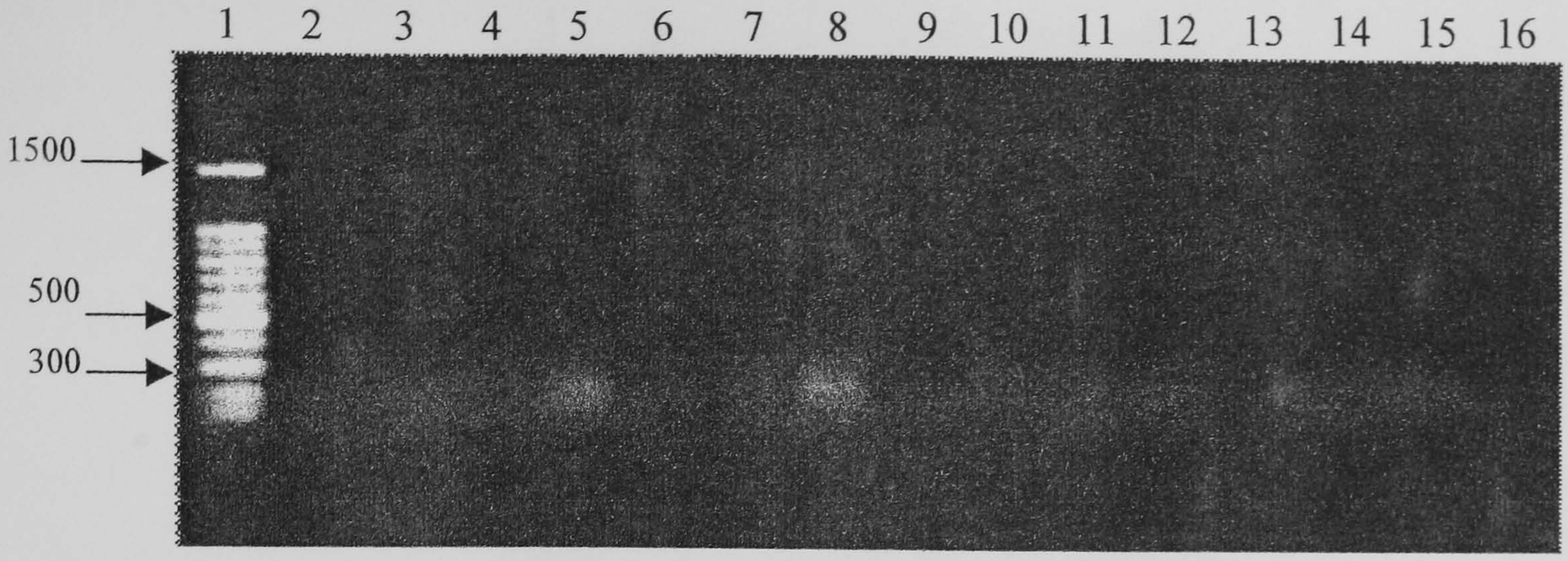


Figure 14.3c. Calliphorin amplicons from *C. vicina* pupae. Lane 1 contains 100bp DNA ladder. Lanes 2-15 calliphorin PCR products early to late aged pupal RNA samples. Lane 16 contains the negative PCR control (water).

As Figure 14.3 illustrates, calliphorin mRNA is being synthesised during the pupal phase as the presence of PCR products. The negative controls showed again that the cDNA samples synthesised in this work do not contain any residual genomic DNA.

14.2.2 Arylphorin Receptor

To amplify part of the arylphorin receptor gene one set of primer pairs utilised by Burmester and Scheller (1995) was utilised. The forward and reverse primers in this work are those nominated R1 and R6 in the work of Burmester and Scheller (1995). The theoretical amplicon size from this primer pair set is 600bp.

According to the manufacturer of the primers (Invitrogen) the annealing temperatures are 54 and 58°C (forward and reverse respectively). A thermal gradient PCR was therefore carried out with annealing temperatures ranging from 50 to 60°C. The PCR yielded negative results when run on an agarose gel (not illustrated). Another gradient PCR was carried out using annealing temperatures ranging from 45-60°C. Products from this are illustrated in Figure 14.4.

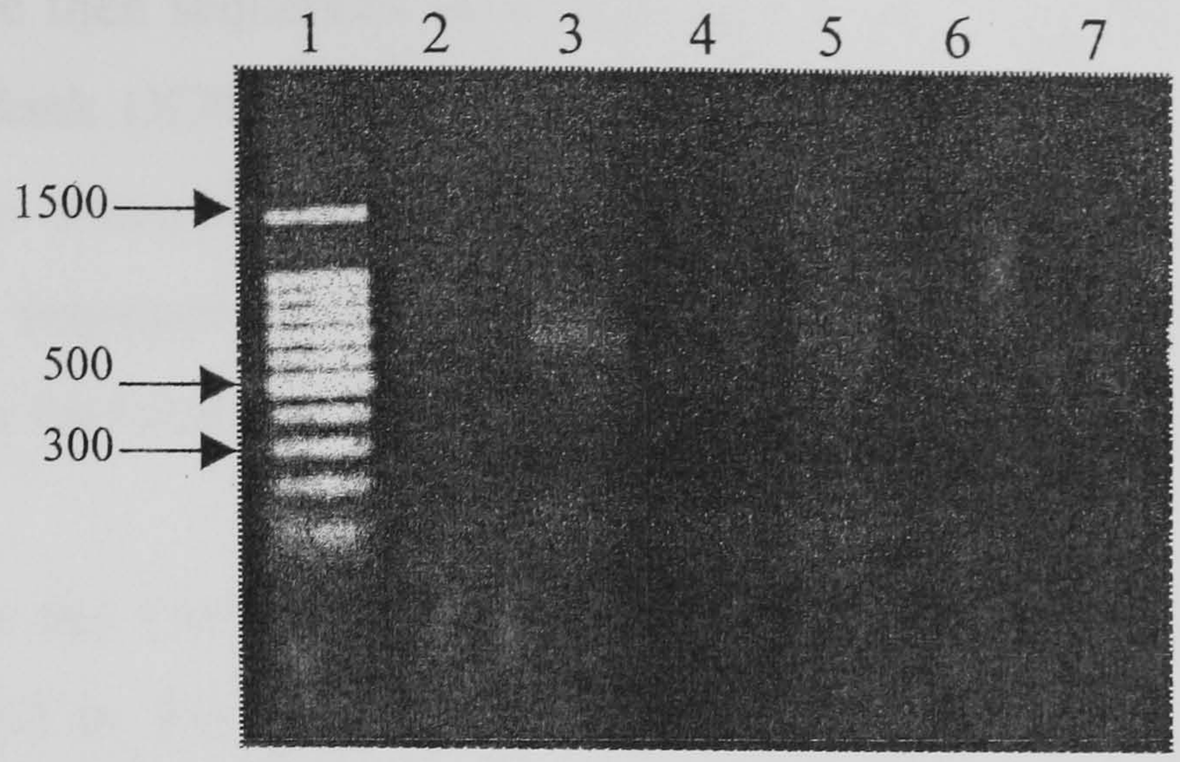


Figure 14.4. Gel picture of thermal gradient PCR of *C. vicina* arylphorin receptor products. Lane 1 contains 100bp DNA ladder. Lanes 2-7 contain amplified products with PCR annealing temperatures 45, 46.3, 49.2, 53.9, 57.7, 60°C.

This clearly shows that the optimum annealing temperature for these primers is between 46-47°C. There are faint bands in other positions but Lane 3 in Figure 14.4 shows the only clear distinct band.

To assess whether it was viable to continue with this gene with the *C. vicina* pupal samples, a PCR was also carried out on cDNA using the annealing temperature of 47°C established. Amplicons are presented in Figure 14.5.

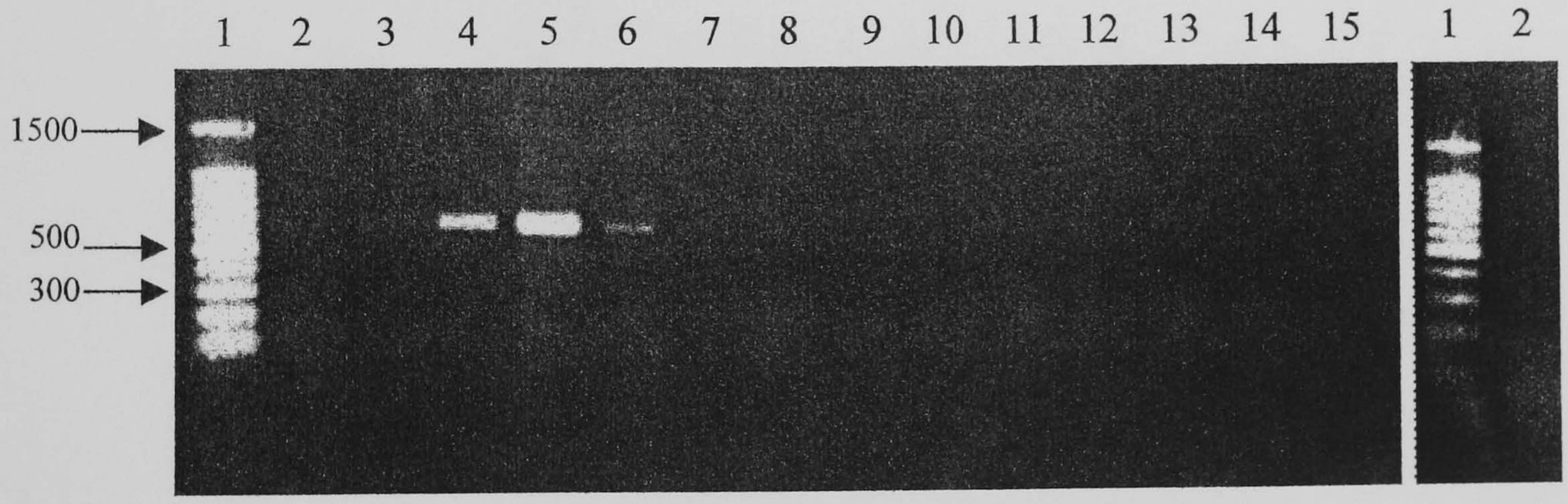


Figure 14.5. Amplicons of arylphorin receptor PCR using cDNA from *C. vicina* pupal samples. Lane 1 contains 100bp DNA ladder. Lanes 2-15 contain early through to late pupal stage samples. On the separate gel Lane 1 contains the 100bp ladder and Lane 2 the negative PCR control.

From this it appears that arylphorin receptor mRNA is being synthesised during the pupal stage of the *C. vicina* lifecycle.

PCR products were then sequenced as in Section 5.6 and compared to the sequence deposited in GenBank (X79100) by Burmester and Scheller (1995). Alignment of these sequences is presented in Figure 14.6. Base numbering is relative to the complete X79100 sequence. Each sequence (test 1, 600bp and test 2, 501bp) has 100% homology to the GenBank X79100 sequence.

Using BLAST on the GenBank sequences no close matches were found for the sequences produced in this work apart from X79100 and a clone from the same authors X79101 (592/600bp similarity and an Expect value of 0.0). A storage binding protein of *Sarcophaga peregrina* (Robineau Desvoidy) (44/51bp; E=0.1 and a later section of 58/67bp; $E=2e^{-06}$) was also located.

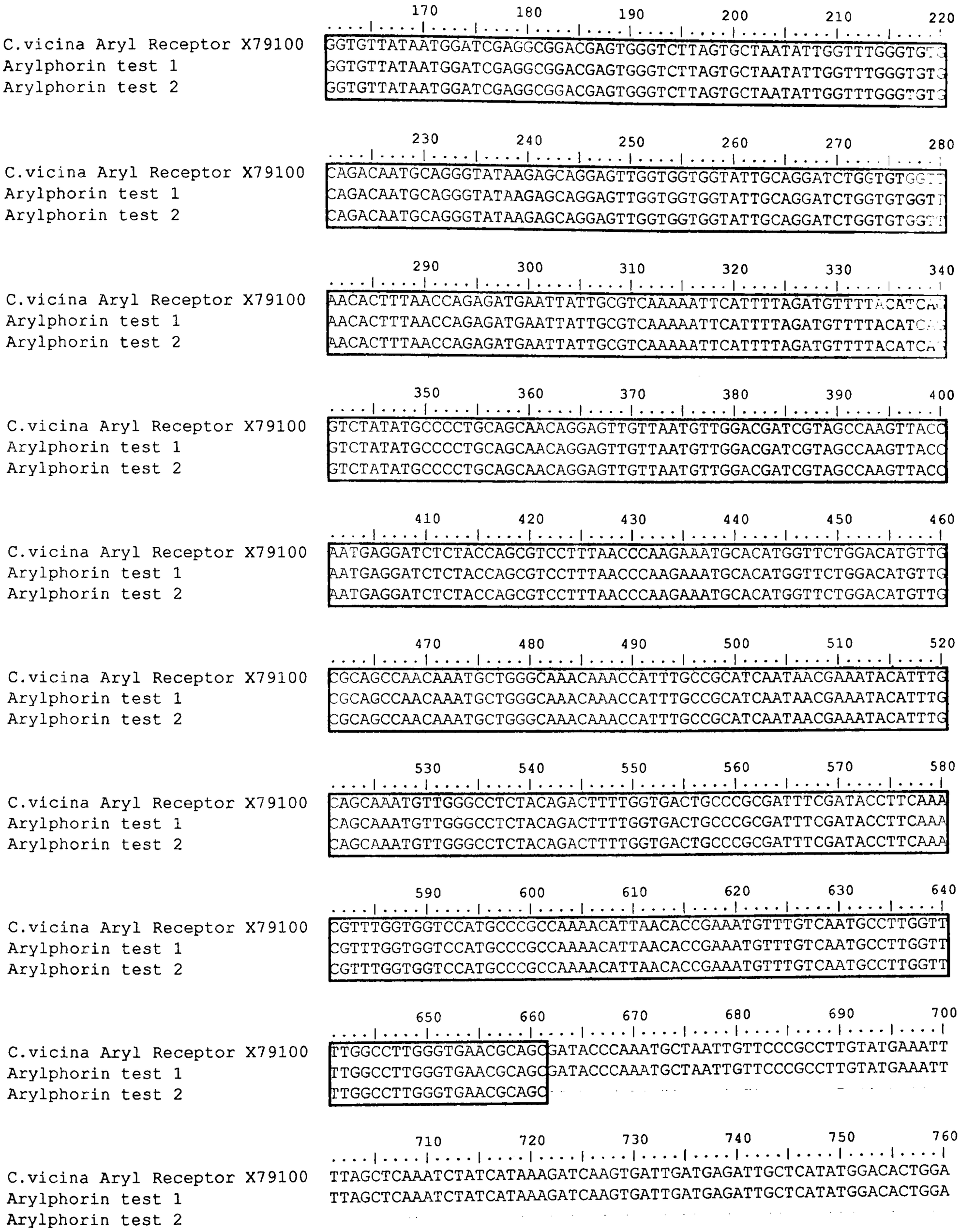


Figure 14.6. Alignment of *C. vicina* arylphorin receptor sequences. The sequences established in this work are labelled Test 1 and 2. Numbering of bases is relative to the complete X79100 sequence. The black outline highlights identical nucleotides between the sequences.

14.2.3 LSP-2

This set of primers was designed using Primer 3 software on GenBank sequence accession number U89789. The theoretical amplicon size was 367 basepairs.

A thermal gradient PCR was conducted between 50-60°C to establish the optimum annealing temperature for this primer pair.

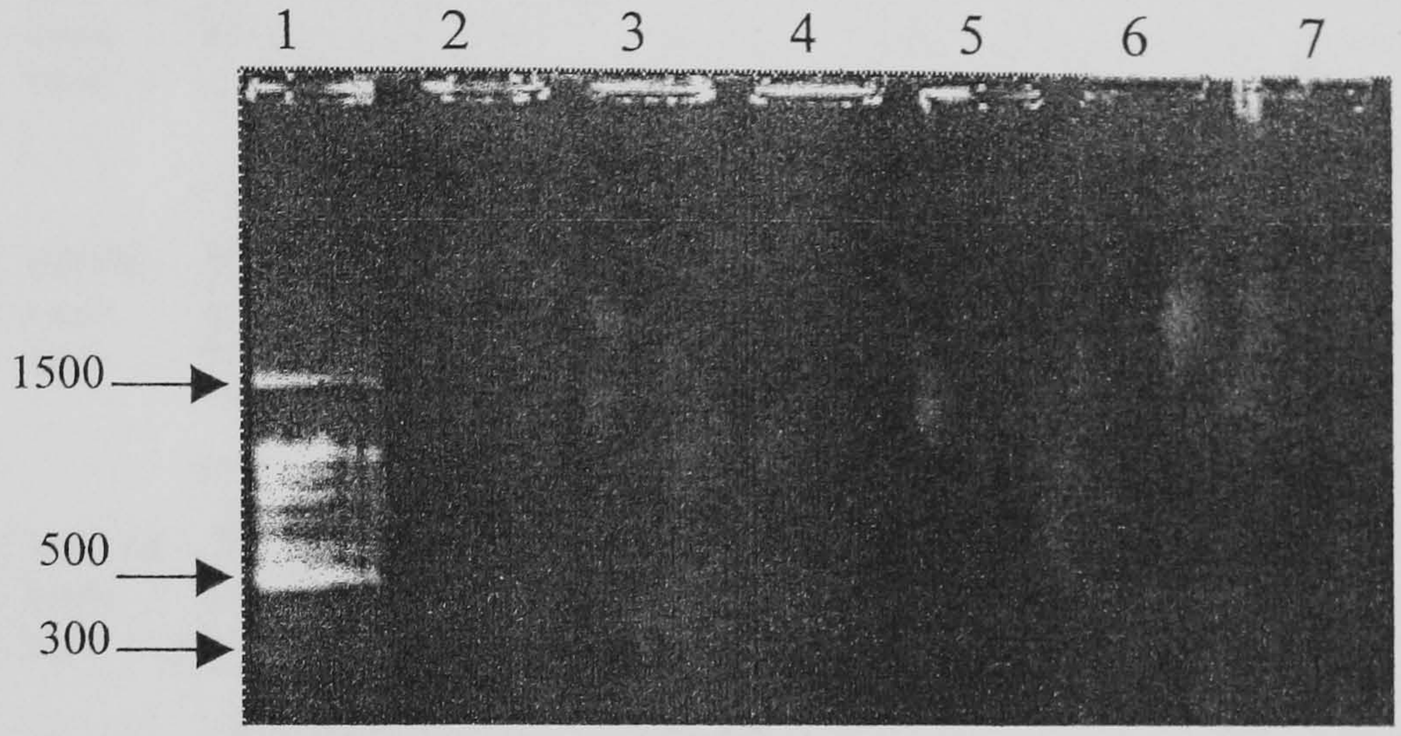


Figure 14.7. Gel picture of thermal gradient PCR of *C. vicina* LSP-2 products. Lane 1 contains 100bp DNA ladder. Lanes 2-7 contain amplified products with PCR annealing temperatures 50, 51.7, 54.3, 57.7, 59.3 and 60 °C.

Annealing temperatures of 57.7°C and above do not above produce amplicons for this primer pair.

Pupal cDNA samples were also subjected to PCR with these primers to assess whether LSP-2 was present at all during the *C. vicina* pupal phase. The PCR was conducted with an annealing temperature of 55°C, slightly higher than the 54.3°C of the thermal gradient. If the primer pair amplifies at this annealing temperature actin and LSP-2 amplifications could be performed simultaneously during later work, thus increasing throughput. The resulting amplicons are shown in Figure 14.8.

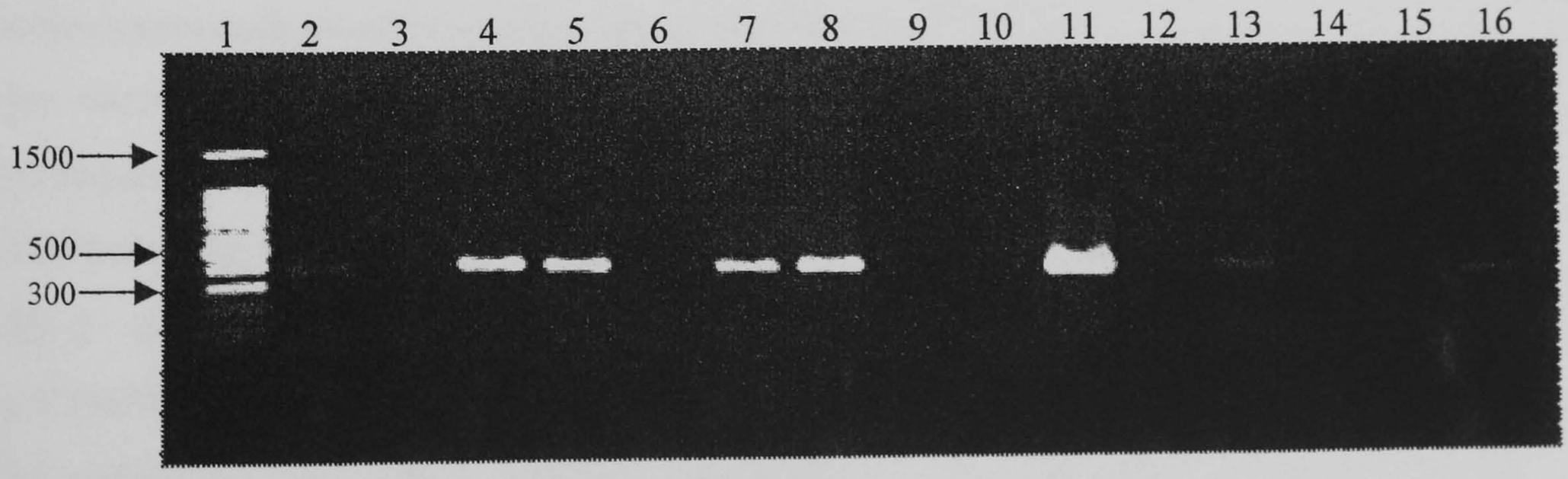


Figure 14.8. Amplicons after amplification of *C. vicina* cDNA samples with primers designed for a LSP-2 gene region. Lane 1 contains a 100bp DNA ladder. Lanes 2 to 16 contain LSP-2 amplicons for early through to late pupal cDNA samples.

LSP-2 is present in the *C. vicina* pupal cDNA samples and so would be used in further research. The annealing temperature of 55°C is satisfactory for this primer pair.

Two PCR products were then sequenced and compared to GenBank sequence U89789 (Figure 14.9).

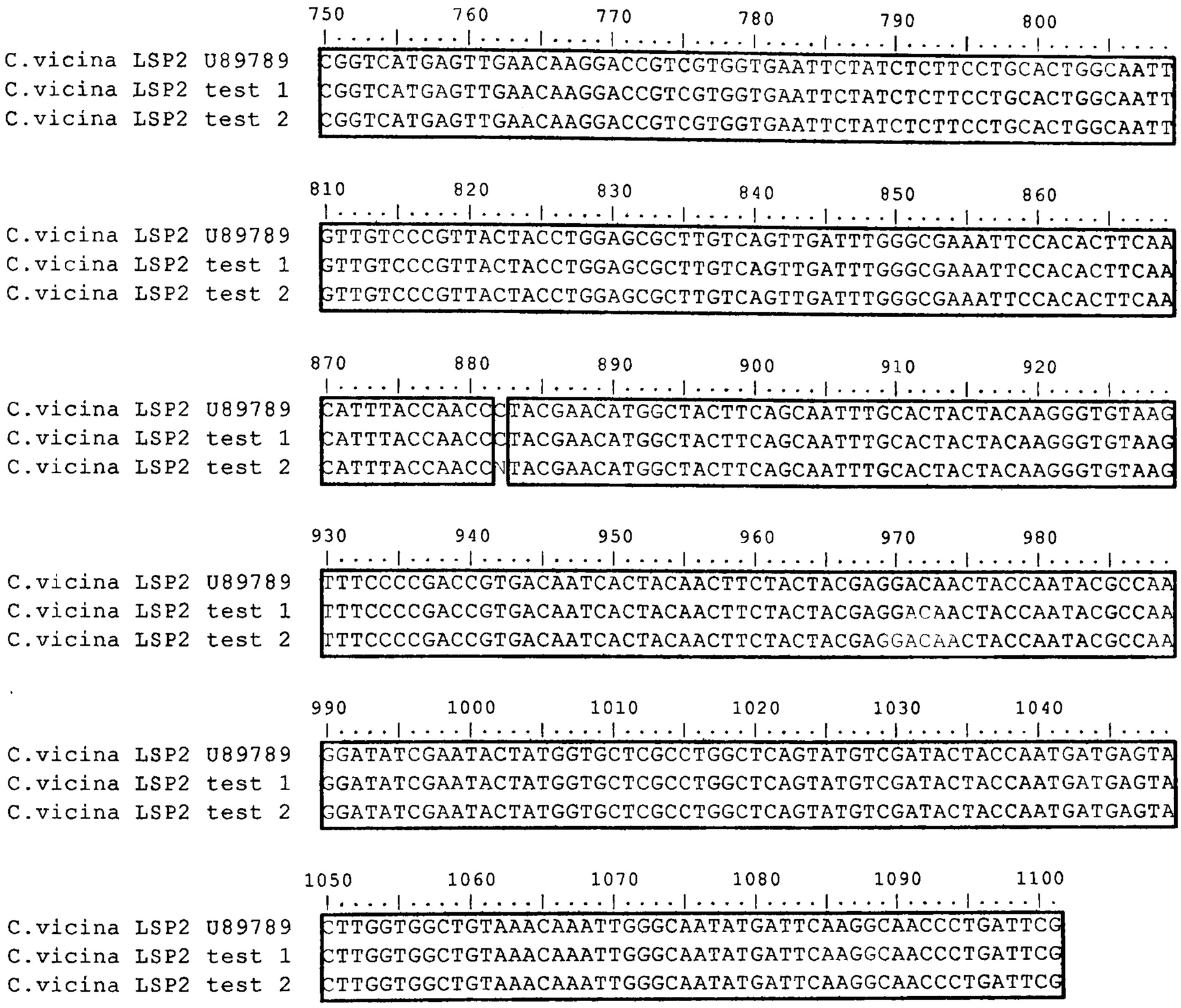


Figure 14.9. Sequence alignment of partial LSP-2 region in *C. vicina* DNA samples with GenBank U89789. Numbering is relative to complete LSP-2 sequence U89789. Black outline surrounds identical nucleotides in different sequences.

Test sequence 1 has a homology of 100% with the GenBank U89789 sequence and test sequence 2 has a homology of 99.7% with the GenBank sequence.

After using the BLAST facility on the NCBI website (as described in Section 5.7) the LSP-2 'test' sequences also had similarities with *M. domestica* hexamerin (AY256681; 132/166bp; $E=4e^{-06}$).

The calliphorin, arylphorin receptor and LSP-2 primers designed for this study or from the literature all appear to be successful in amplifying the correct partial gene regions and therefore would be used in this work to examine their use as temporal markers for the *C. vicina* pupal phase.

Chapter 15

Design of Primers - Differential Display

Another approach for location of variation in gene expression is to utilise differential display techniques. Differential display amplifies cDNA from different conditions (e.g. disease/control or different timepoints) using a 3' poly-T primer and a 5' random primer as described by Liang and Pardee (1992) and Liang *et al.* (1993). After separation and visualisation of the amplicons on a gel any differences between samples may be considered differentially expressed genes (DEGs).

When initially described by Liang and Pardee (1992) amplicons were run on denaturing a polyacrylamide gel. There is a kit - GeneFishing™ DEG 100 Kit (SeeGene, USA) which is based upon differential display techniques and the amplicons are separated on agarose gels. The primers it contains are annealing control primers (ACP, patent pending). These primers minimise the number of false positives normally associated with the use of random primers (Section 3.1.6). This is possible because the primers consist of three distinct regions. The core-targeting region (at the 3' end) binds randomly during the first cycle of PCR when the annealing temperature is low (therefore during less stringent annealing conditions). The middle portion of the oligonucleotide, known as the 'regulator' does not anneal at the temperatures of the first cycle and this inhibits binding of the 5' 'universal' region to the template cDNA. The remaining PCR cycles contain a higher annealing temperature thus increasing the stringency. At this temperature the universal sequences of the 5' end of the primers will bind to each other only allowing amplification of the fragments created in the first cycle of PCR. Random priming to the template cDNA is not theoretically possible in later cycles. This PCR is reproducible, eliminating the problem that exists with other techniques using random primers.

15.1 Materials and Methods

15.1.1 Pupal Samples, RNA Extraction

The RNA extractions carried out as described in Section 12.4 were used in this differential display work. The cDNA synthesis methodology in Section 12.8 could

not be used with this kit as the ACP primers need to be incorporated at the cDNA synthesis stage to act as the binding site for the ACP primers in the subsequent PCR.

15.1.2 cDNA Synthesis

cDNA was synthesised using the dT-ACP1 primer from the Gene Fishing DEG Kit (GeneHunter) along with the Omniscript™ Reverse Transcriptase kit (QIAGEN) according the instructions from the Gene Fishing DEG Kit (GeneHunter). Total RNA (3µg) was added to a 1.5ml tube along with 2µl of 10µM dT-ACP1 primer (Gene Fishing Kit, SeeGene). Each sample was made up to a volume of 15µl with ddH_2O. Samples were then incubated at 80°C for 3 min after which the tubes were chilled on ice for 2 min and reagents briefly spun. The following were then added, 2µl of 10X Buffer, 2µl of 5mM dNTP and 1µl Omniscript reverse transcriptase (QIAGEN) to a make a final volume of 20µl per reaction. Tubes were then incubated at 42°C for 90 min, after which they were heated for 2 min at 94°C to inactivate the enzyme and chilled on ice for 2 min.

cDNA samples were then purified using the GFX PCR and Gel Band Kit (Amersham Biosciences) according to manufacturer's instructions for PCR product purification (Section 5.5). All samples were eluted off the spin column with 100µl ddH_2O as suggested in the GeneFishing Protocol.

15.1.3 cDNA Quantification

Samples were quantified on the NanoDrop® ND-1000 spectrophotometer and concentrations were calculated as described in Section 12.9.

15.1.4 GeneFishing™ PCR

To locate DEGs it is critical that the same amount of cDNA is added to each reaction. The GeneFishing protocol recommends 50ng of cDNA for each PCR reaction. The appropriate volume of cDNA was added to a 0.2ml PCR tube along with 5µl 10X GeneAmp Buffer without $MgCl_2$ (Applied Biosystems), 5µl of 25mM $MgCl_2$, 2µl of 5µM arbitrary ACP, 1µl of 10µM dT-ACP2, 2ml of 5mM dNTP (Promega), 0.5µl of AmpliTaq Gold® (5U/µl, Applied Biosystems, USA) and made up to a final volume of 50µl with ddH_2O.

After an initial 94°C for 12 min to activate the *Taq* polymerase enzyme, the cycling parameters were 94°C for 3 min; 50°C for 3 min; 72°C for 1 min; 40 cycles of [94°C

for 40s; 65°C for 40s; 72°C for 40s]; a final extension period of 72°C for 5 min and 4°C to finish. Several arbitrary ACP primers are supplied with the kit. Two of these ('g' and 'h') were included in separate PCR experiments on all the cDNA samples. Samples were run on a 2% TAE agarose gel stained with ethidium bromide and visualised under UV light.

15.1.5 Low Melting Point Gel

When any DEGs were observed on the agarose gel then the equivalent samples were electrophoresed on a low melting point gel. Bands were excised using a clean scalpel and the band was purified using the GFX PCR and Gel Band Purification Kit (Amersham) using the protocol for gel band excision (see Section 5.5) and eluted off the spin column with 20µl ddH_2O.

15.1.6 Sequencing

DEGs were sequenced on the 310 Genetic Analyser (Applied Biosystems, USA) as described as Section 5.6. Forward and reverse samples were compared and the consensus sequences were compared with other sequences located in GenBank using BLAST. This was to search for homology with other already sequenced gene regions.

15.1.7 Primer Design

From the sequences produced primers were designed using Primer3 software. These primers were then tested on *C. vicina* DNA as part of a thermal gradient PCR to gauge the optimum annealing temperature of each primer pair. DNA was amplified using a range of annealing temperatures from 45 to 60°C. Amplicons were separated on an agarose gel, stained with ethidium bromide and visualised under UV light.

15.1.8 Sequencing

Once the designed DEG primers had been tested, the amplicons were sequenced to ensure that the primers are amplifying the region intended. Sequencing was carried out on the 310 Genetic Analyser as described in Section 5.6.

15.2 Results

The amplicons from the primer 'g' and 'h' PCR reactions are illustrated in Figure 15.1 and 15.2.

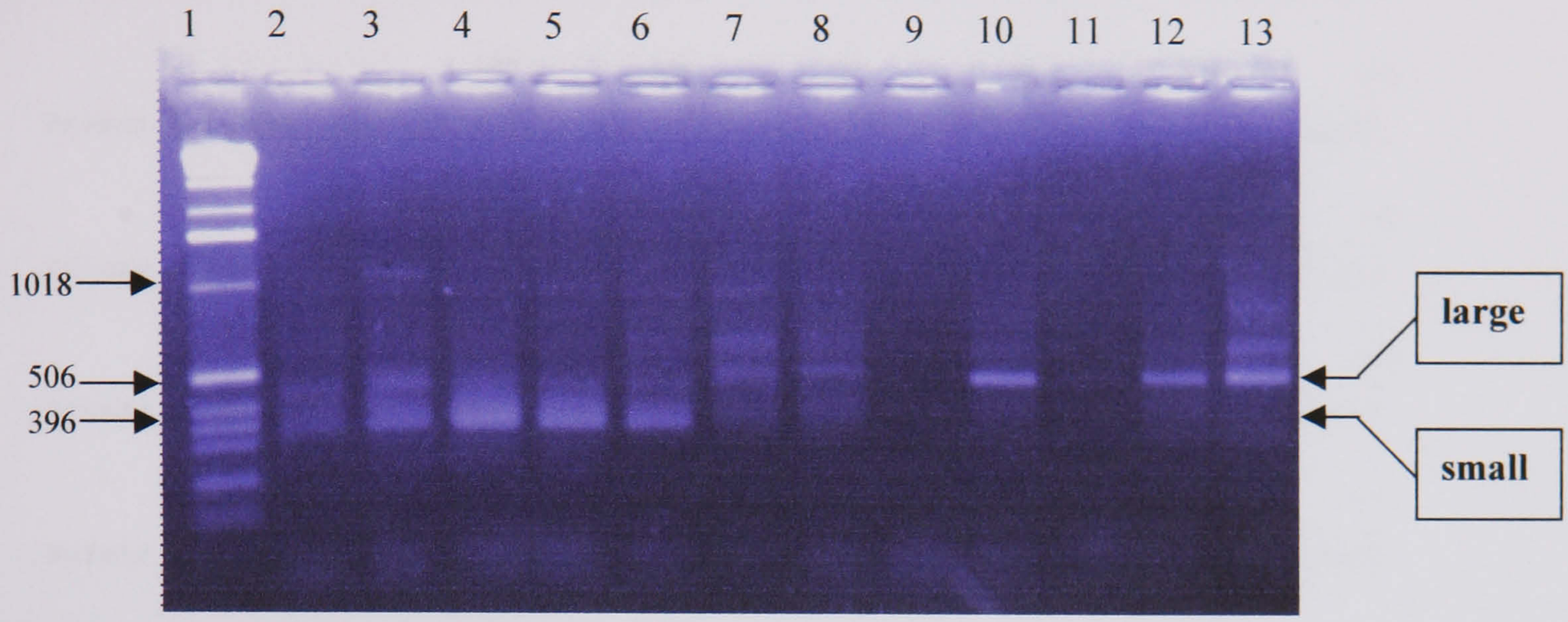


Figure 15.1. Amplicons produced from GeneFishing PCR using Primer 'g' as the arbitrary primer. The two main bands are labelled large and small to demonstrate which bands were excised and sequenced. Lane 1 contains a 1KB DNA ladder. Lanes 2-13 contain cDNA samples (2880, 3360, 3840, 4320, 5760, 6240, 6720, 7200, 9120, 9600, 10080 and 10560 ADH)

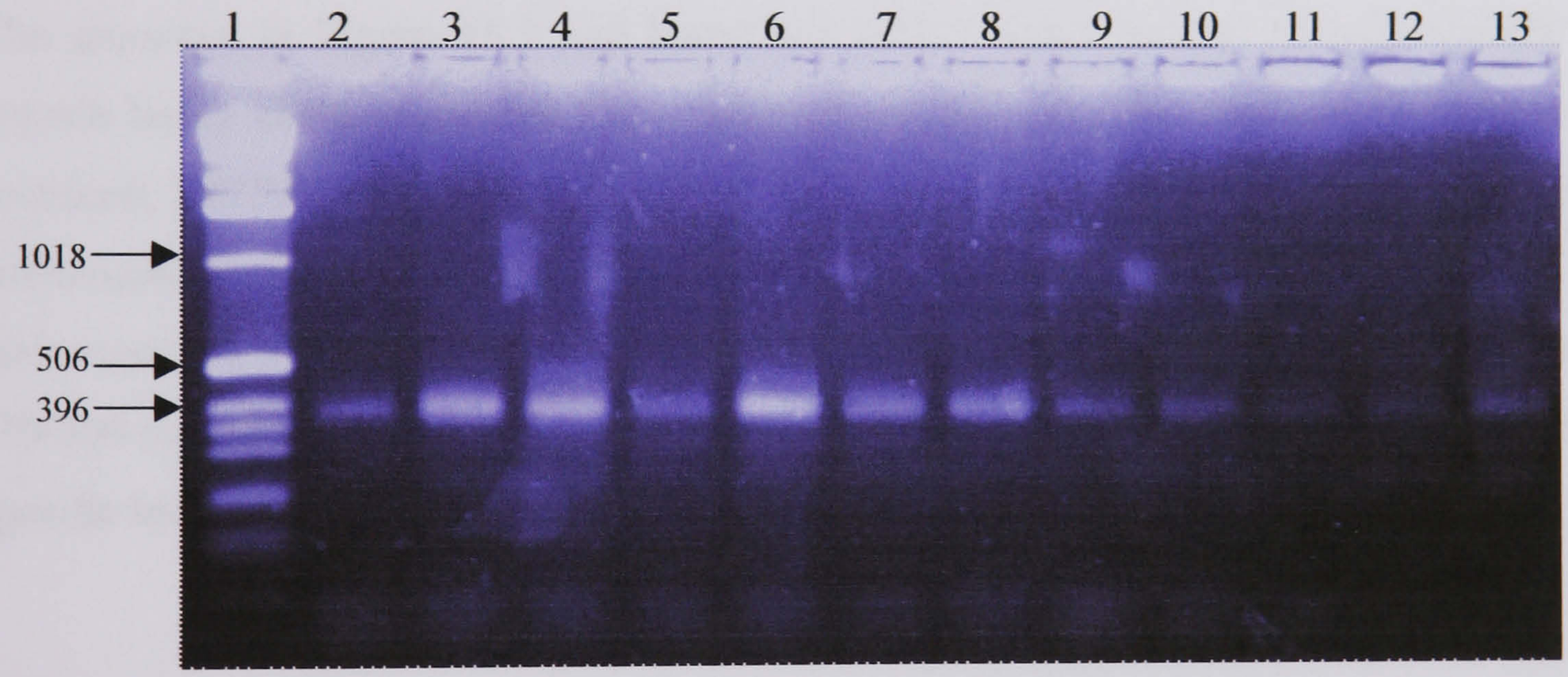


Figure 15.2. Amplicons produced from GeneFishing PCR using Primer 'h' as the arbitrary primer. Lane 1 contains a 1KB DNA ladder. Lanes 2-13 contain cDNA samples (2880, 3360, 3840, 4320, 5760, 6240, 6720, 7200, 9120, 9600, 10080 and 10560 ADH)

The large and small 'g' and 'h' amplicons were then sequenced and these are presented in Figures 15.3, 15.4 and 15.5. These sequences were then compared with the samples deposited in GenBank to examine for sequence homology.

```
                    10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Large      GGGGGGCTCCGATGCCCAAAAGACCTCCGCAAATCCGAACGTCGCATTAAGGAATTGAGC

                    70        80        90        100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Large      TTCCAATCTGAAGAAGACCGCAAGAACCACGAACGTATGCAAGACTTGGTTGACAAACTC

                    130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Large      CAACAAAAGATCAAGACATACAAGAGGCAAATCGAAGAAGCCGAAGAAATCGCCGCCCTC

                    190       200       210       220       230       240
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Large      AACTTGGCCAAATTCCGCAAAGCCCAACAAGAACTTGAAGAAGCCGAAGAACGTGCCGAT

                    250       260       270       280       290       300
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Large      ATGGCTGAACAAGCCATCAGCAAATTCCGTGCCAAGGGACGTGCCGGTTCCGTTGGCCGT

                    310       320       330       340       350       360
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Large      GGTGCCAGCCCCGCGATTTAAATTACGCAAAATGGCACTGTAAATGATTCACACGGTCCA

                    370       380
                    ....|....|....|....|...
Primer G Large      CATAGAGATTCAATTAGCAAACC
```

Figure 15.3. Sequence of large fragment produced from amplification of *C. vicina* cDNA using random primer g.

The sequence in Figure 15.3 had homology with various insects' muscle specific myosin heavy chain sequences deposited in GenBank. *Drosophila hydei* Sturtevant sequence, X77570, had 246/284 nucleotide matches, with an expect value of $6e^{-67}$; *D. melanogaster*, NM1615192, matched 264/310bp (expect value = $5e^{-61}$) and *D. melanogaster*, M13360, sequence from cDNA from larval/adult thoracic muscle has 259/304 nucleotides that match and an expect value of $8e^{-60}$. No Calliphoridae muscle specific heavy chain sequences are deposited in GenBank for comparison.

```
                        10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Small  GGGGGCTCCGATGCCCATCTAATGGTTAAGGATGTTAAAATCTATCATGATGACAATATT

                        70        80        90        100       110       120
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Small  AAAATGCCTCTTAGCCATTTGTACTAAATAATTGAATGAATGCGATGACTACAACTGAAA

                        130       140       150       160       170       180
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Small  GTGATTACTGTGTCTGTGCTCTTGGACTTGATTCCCTCCTCCCCCGATTATTTTAAATTT

                        190       200       210       220       230       240
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer G Small  CTAAATATATATTTTGGTTAAATTGGATAAAGTGTTGGCAATAAATGCCTTAAAAAAGCT

                        250
                ....|....|....|.
Primer G Small  TAAAAAAAAAAAAAAA
```

Figure 15.4. Sequence of small fragment produced from amplification of *C. vicina* cDNA using random primer g.

There are only two sequences within GenBank that show any significant homology to the sequence in Figure 15.4 and these are both *C. vicina* arylphorin receptor sequences, X79101 and X79100 with 222/232 nucleotide matches and expect values of e^{-100}. These sequences were used in the previous chapter to produce primers for the arylphorin receptor. Whilst this DEG has not provided a new gene to test for temporal variation, it does provide support for examining the arylphorin receptor gene region.

```
                        10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer H        GGGGGGCAGCCCACCAAGGTTGCCGGAAAGAAACCTACCATAGGCTGGCTCCAGGCCACC

                        70        80        90        100       110       120
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer H        GGCAAATCTGCTGCCAAGAAGCCCGCTCAAAAAGCCACCGGCGAATCCAAGAAAGTAAAT

                        130       140       150       160       170       180
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer H        TAACATCCAAAATTTGCTGCAGTGTTTTTGAGTGTTGAGTTTTTCTTAGGATTTAAGTGA

                        190       200       210       220       230       240
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer H        GACGTGTGTGTTTATACAACTAATAACAAACAATTTTATTATACAAAATCTGTGTATAGA

                        250       260       270       280       290
                ....|....|....|....|....|....|....|....|....|....|....|....|
Primer H        ATTCAACGTTGATAAAAATGAAGTGAAAACATTTTACATTAAAAAAAAATCTAAA
```

Figure 15.5. Sequence produced by amplification of *C. vicina* cDNA using random primer h.

This sequence has poor homology with anything in GenBank. The best match was to a *Bacteroides fragilis* glucose-1-phosphate thymidyltransferase sequence. This had 23/23 +/- bp match (the *C. vicina* sequence matches the complementary strand of the sequence in GenBank) with an expect value of 0.05. This identity is not close enough to make any inference as to the function of this DEG.

The sequences were used as templates for designing primers using Primer3 software. These primers are presented in Table 15.1. As the Primer g small sequence has been used previously (Chapter 14) it was not included. The 'g' (large) amplified fragment should be 199bp due and 'h' should be 100bp.

Table 15.1. Primer sequences designed for amplification of the DEGs established as potential markers for *C. vicina* pupae.

Primer Name	Sequence
g – Forward	5' CGAACGTCGCATTAAGGAAT 3'
g – Reverse	5' ACGTTCTTCGGCTTCTTCAA 3'
h - Forward	5' CAAATCTGCTGCCAAGAAGC 3'
h - Reverse	5' AACACACACGTCTCACTTAAAT 3'

These primers were utilised in a thermal gradient PCR to establish the annealing temperatures and the amplicons visualised on an agarose gel (Figure 15.6).

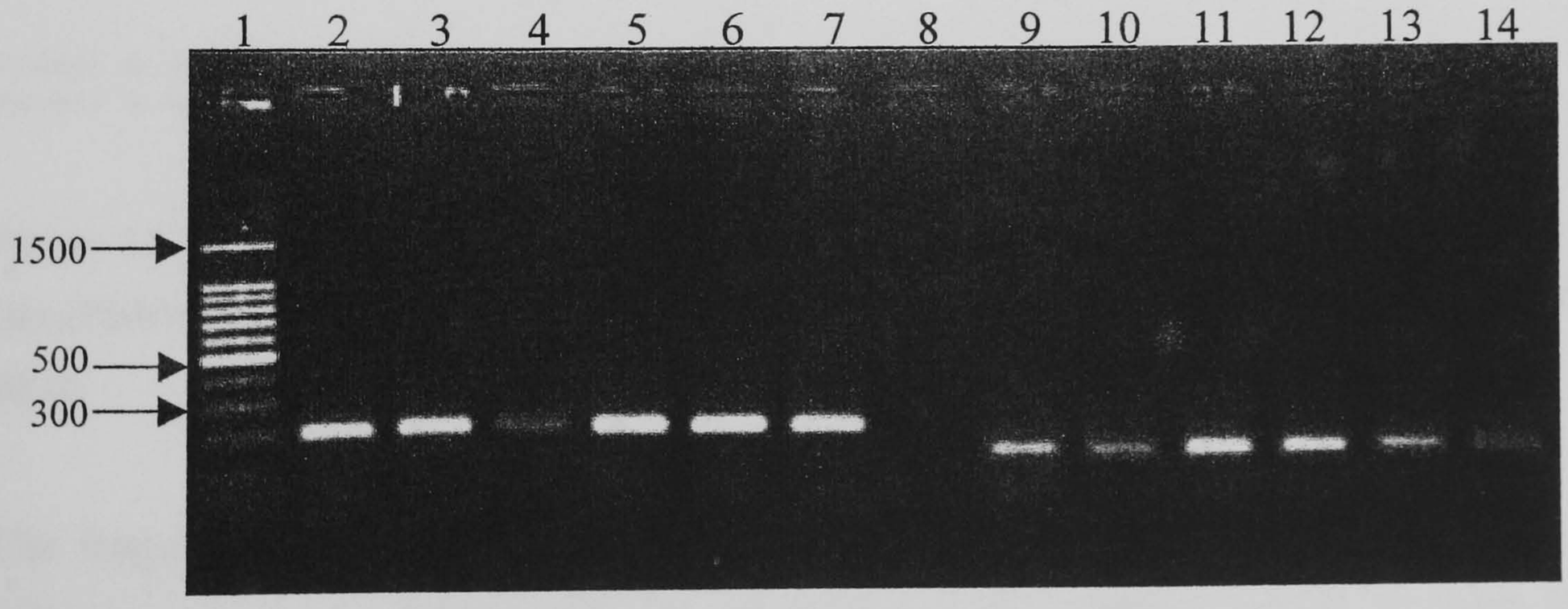


Figure 15.6. Amplicons from a thermal gradient PCR of g and h primer pairs on *C.vicina* cDNA. Lane 1 contains 100bp DNA ladder. Lanes 2-7 contain amplicons for g primers. Lanes 9-14 contain amplicons for h primers. The annealing temperatures were 45, 46.3, 49.2, 53.9, 57.7 and 59.7°C.

The amplicons are of the expected length and appear to work at all temperatures up to approximately 60°C. For the g primers the two lowest temperatures (45 and 46.3°C) there is slight shadowing of a second band beneath the correct amplicon. It is not advisable therefore to use the lower annealing temperatures for these primers.

These amplicons were then sequenced and compared to the sequences from Figures 15.3 and 15.5. The primer g sequence had 100% homology to the sequence the primers were designed from (Figure 15.7).

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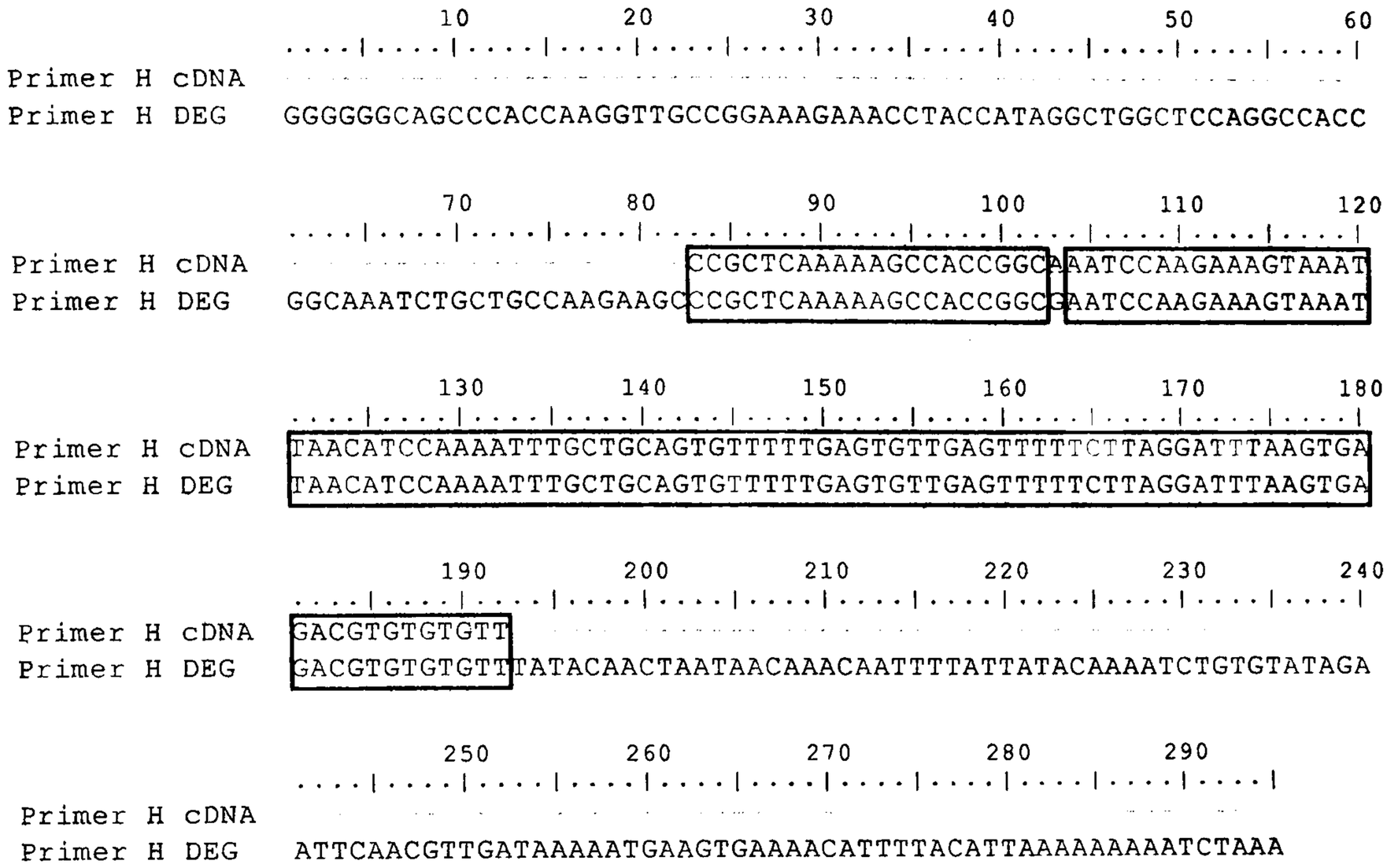

Figure 15.8. Alignment of the DEG produced when using the h primer pair from the GeneFishing kit with the amplicon produced from the new primers designed from the DEG.

Both of these DEG designed primer pairs would both be used in further examination to examine their use as potential molecular markers for age determination during the pupal phase.

Chapter 16

Quantification of gene expression I

Preliminary Quantification

To assess the temporal expression of the genes chosen as potential markers the amount of marker cDNA at various pupal timepoints (and thus mRNA) was quantified. This was done by quantifying the amount of amplified product after PCR. Theoretically the amount of PCR product is proportional to the starting amount of template cDNA. The fragments were separated and quantified using capillary electrophoresis. These quantities, after normalisation against the housekeeping gene actin, were compared between samples throughout the pupal stage to note any changes in relative gene expression.

As discussed in the previous two sections the following gene regions were amplified and qualified, LSP-2, calliphorin, arylphorin receptor and the differential display regions (nominated 'primer g' and 'primer h').

16.1 Materials and Methods

16.1.1 Samples

cDNA samples were as described in Chapter 12.

16.1.2 LSP-2 PCR and Purification

Separate actin and LSP-2 PCR reactions were set up. Each reaction contained 0.5µl (10µM) of each primer, 12.5µl Readymix™ RedTaq™ PCR Reaction Mix (Sigma, UK), approximately 50ng cDNA and the appropriate volume of ddH_2O to a final volume of 25µl.

The samples were amplified on GeneAmp® PCR System 9700 Thermal Cycler (PE Applied Biosystems, USA). The following cycles were run ~ 94°C for 2 min; 35 cycles of [94°C for 30s; 55°C for 45s; 72°C for 90s]; 72°C for 7 min; 4°C to finish.

Equal volumes of actin and LSP-2 PCR products for each experimental condition were pooled before being purified by the GFX PCR DNA and Gel Band Purification

spin column kit (Amersham Biosciences, UK) as described in Section 5.5. Samples were eluted in 30µl ddH_2O.

Samples were then loaded onto DNA 7500 chips for quantification with the 2100 Agilent Bioanalyser. The Bioanalyser software compares chromatogram peak area to peak area of a known standard to calculate DNA concentration. Duplicated sample chips were run.

Pooling the actin and LSP-2 samples eliminated any differences in spin column efficiency and chip-to-chip variation. Final concentrations of LSP-2 products could then be compared to actin concentrations.

16.1.3 Arylphorin receptor, primer g, primer h and calliphorin PCR and Purification

These genes were all amplified in separate thermal cycling reactions. The reaction reagents were as for actin/LSP-2 PCR reactions in Section 16.1.2. Each reaction contained 0.5µl (10µM) of each primer, 12.5µl Readymix™ Red Taq™ PCR Reaction Mix (Sigma, UK), approximately 50ng cDNA and the appropriate volume of ddH_2O to a final volume of 25µl. Actin was also included as this series of amplifications occurred separately to the LSP-2 quantifications and therefore to eliminate any variation between reagents the genes were normalised to the actin quantified simultaneously.

As established in Section 14.2.2, the optimum annealing temperature for the arylphorin receptor primer pair was 47°C and so this was the temperature used. All other genes were amplified with an annealing temperature of 55°C. This annealing temperature was applicable to all the remaining primer pairs and allowed these reactions to be run simultaneously on the GeneAmp® PCR System 9700 Thermal Cycler. The other cycling parameters were the same as for actin/LSP-2 (Section 16.1.2).

Arylphorin receptor, primer g, primer h, calliphorin and actin PCR products for each experimental conditions were pooled before purification with the spin column kit as described in Section 5.5. Purified samples were then loaded into DNA 7500 chips (Agilent). The PCR products were run altogether. This is viable as the amplicons

were of different sizes (actin – 320bp; calliphorin – 301bp; arylphorin receptor – 600bp; primer g – 199bp and primer h – 100bp) and were therefore run simultaneously to avoid inter-chip variation. Duplicate chips were run.

16.1.4 Concentration of samples

If PCR samples produced negative or very low quantities for actin and other genes, they were concentrated using size exclusion filters (Centricons YM-100, Millipore) to reduce the volume to 10µl and rerun.

16.1.5 Data Analysis

Average DNA concentrations for each pupal timepoint were calculated. These concentrations were normalised by dividing by the actin concentration for the particular samples and the log of this value was calculated.

16.2 Results and Discussion

The quantification of amplified products theoretically provides an indication of the relative intial amounts of extracted mRNA from the *C. vicina* pupae. Whilst the PCR products could have been separated using agarose gel electrophoresis and quantified by densitometry, it was decided that the Agilent Bioanalyser capillary electrophoresis provided a more efficient method as it uses less sample volume and has greater sensitivity.

Variation within the experiment was minimised by the pooling of different PCR products to avoid differences between efficiencies of spin column purification and to minimise any chip-to-chip variation. It was important to minimise the variation between actin and the other marker genes as they would be normalised to actin.

16.2.1 LSP-2

The Agilent Bioanalyser produces chromatograms as illustrated in Figure 16.1. The samples were run individually and the resultant peaks from all samples can be overlaid as demonstrated in this Figure. As expected the actin and LSP-2 fragments resolved and produced fragments of 367bp and 320bp. Markers (upper and lower) are included in each sample run to allow samples to be compared and thus eliminate for any run-to-run variation.

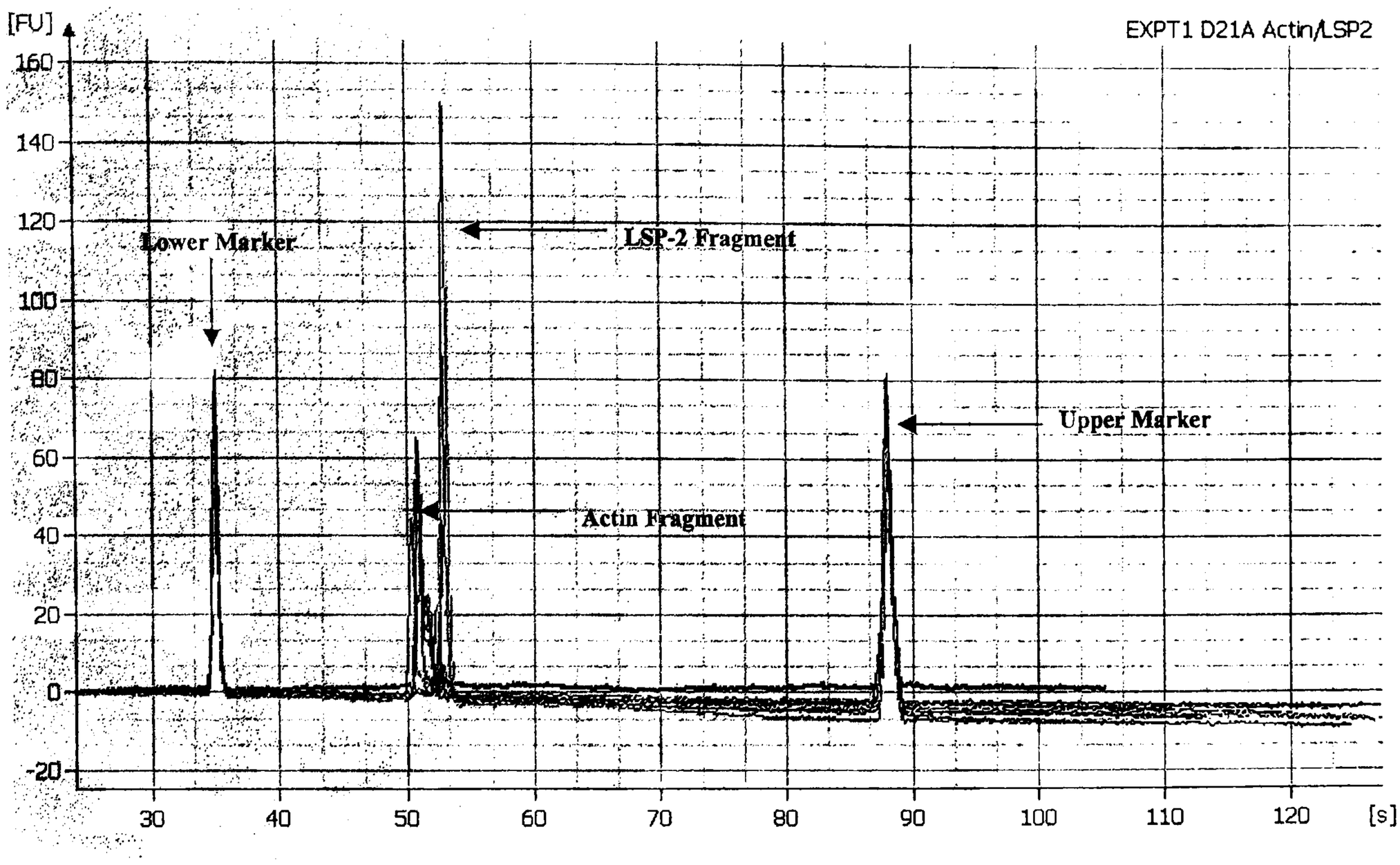

Figure 16.1. Overlay of pupal sample chromatograms of actin and LSP-2 amplicons. The individual peaks are labelled according to the fragment they represent. The lower and upper markers allow comparison to the ladder (run separately, not shown) for sizing of amplicons. Markers are of known DNA concentration and thus comparison of peak area allows calculation of original concentrations of fragments.

The average concentration for each pupal timepoint for actin and LSP-2 was calculated along with the standard deviation. From this the log of the [LSP-2/actin] ratio was calculated and presented in Table 16.1. The nature of these calculations means that positive values indicate greater expression of the LSP-2 gene compared with actin and negative results show less LSP-2 amplification product than actin.

Table 16.1. Average DNA concentrations of actin and LSP-2 after amplification for each pupal timepoint (Day 6/ 2880 ADH third larval instar also included). Average values±standard deviation. The log_{10} of the ratio [LSP-2/actin] is included for each timepoint. ADH values based upon specimens kept at 20 °C.

Day	ADH	Mean Concentration ± s.d. (ng/µl)		Log_{10} [LSP-2 / actin]
		actin	LSP-2	
6 (Larval 3)	2880	3.87±1.88	3.56±0.57	-0.04
7	3360	6.09±6.61	6.26±0.70	0.01
8	3840	4.46±3.25	1.57±1.38	-0.45
9	4320	1.85±1.51	2.12±1.93	0.06
10	4800	3.19±2.08	5.12±3.39	0.21
11	5280	4.45±0.22	3.07±2.08	-0.16
12	5760	1.23±1.34	1.49±1.22	0.08
13	6240	2.94±1.25	2.87±0.81	-0.01
14	6720	2.16±0.76	3.94±1.74	0.26
15	7200	2.58±1.27	4.19±2.49	0.21
16	7680	4.31±2.33	6.19±5.31	0.16
17	8160	3.13±0.13	2.26±0.15	-0.14
18	8640	3.79±2.57	1.44±0.96	-0.42
19	9120	3.15±1.53	0.81±0.65	-0.59
21	10080	7.46±0.00	0.55±0.00	-1.13
22	10560	5.14±1.65	2.00±1.64	-0.41

The Log_{10} [LSP-2 / actin] values were plotted against the appropriate ADH value (Figure 16.2).

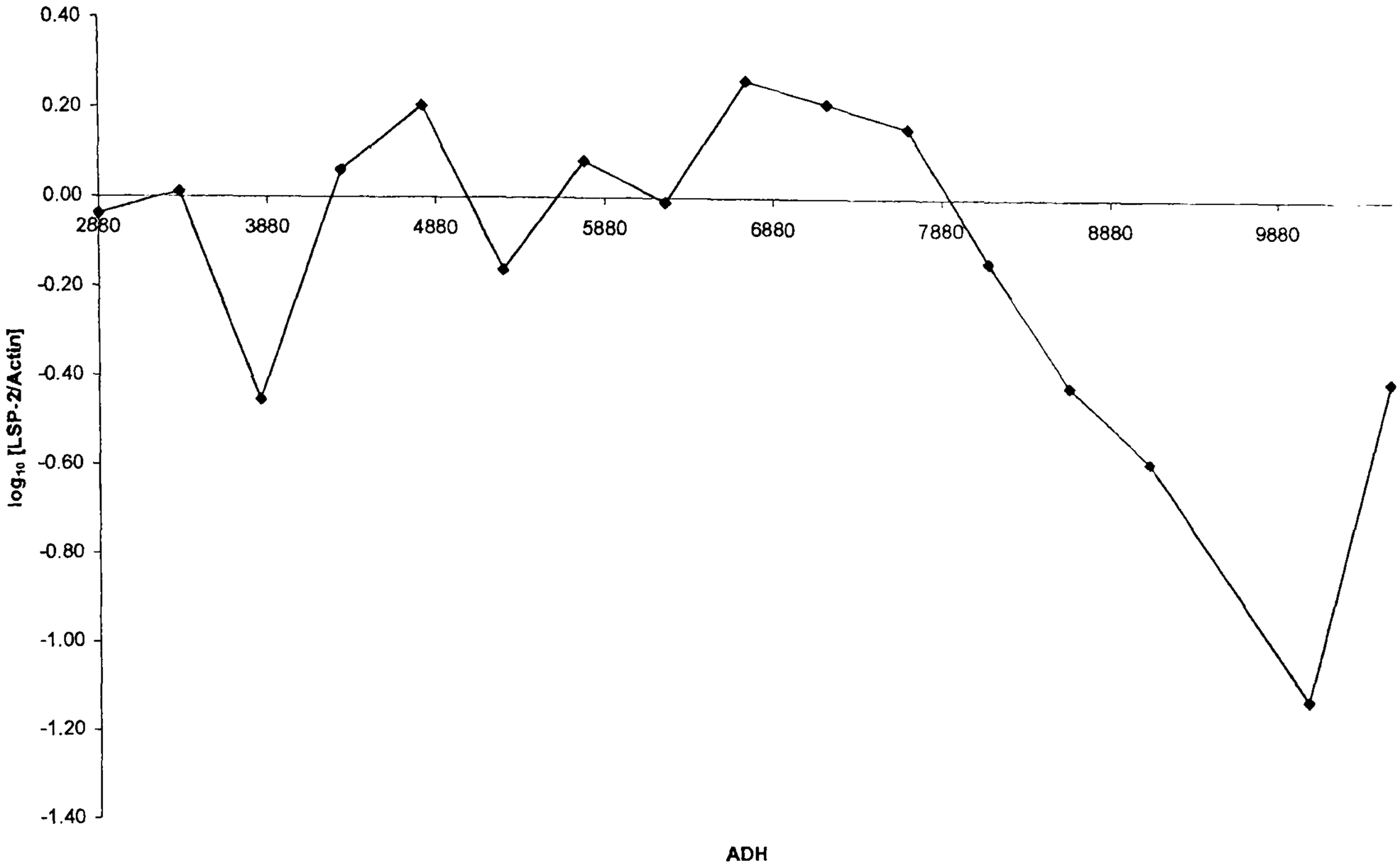

Figure 16.2. Log_{10} [LSP-2 / actin] plotted against ADH for the pupal stage of *C. vicina*.

The graph illustrates that after an initial decrease (3840 ADH), the level of LSP-2 increases over the middle of the pupal phase before declining rapidly after 7680 ADH toward the end of the pupal stage. There is an increase in the amount of LSP-2 at the final timepoint (10560 ADH). This gene could therefore act as a marker to distinguish the early/late pupal phase.

16.2.2 Arylphorin receptor, calliphorin, primer g and primer h

Similar to Figure 16.1, the overlaid graphs produced for the arylphorin receptor, calliphorin, primer g and primer h amplification products are presented in Figure 16.3. Although the calliphorin and actin fragments are similar in size (301 and 320bp), they separate in this capillary electrophoresis system and so can be quantified.

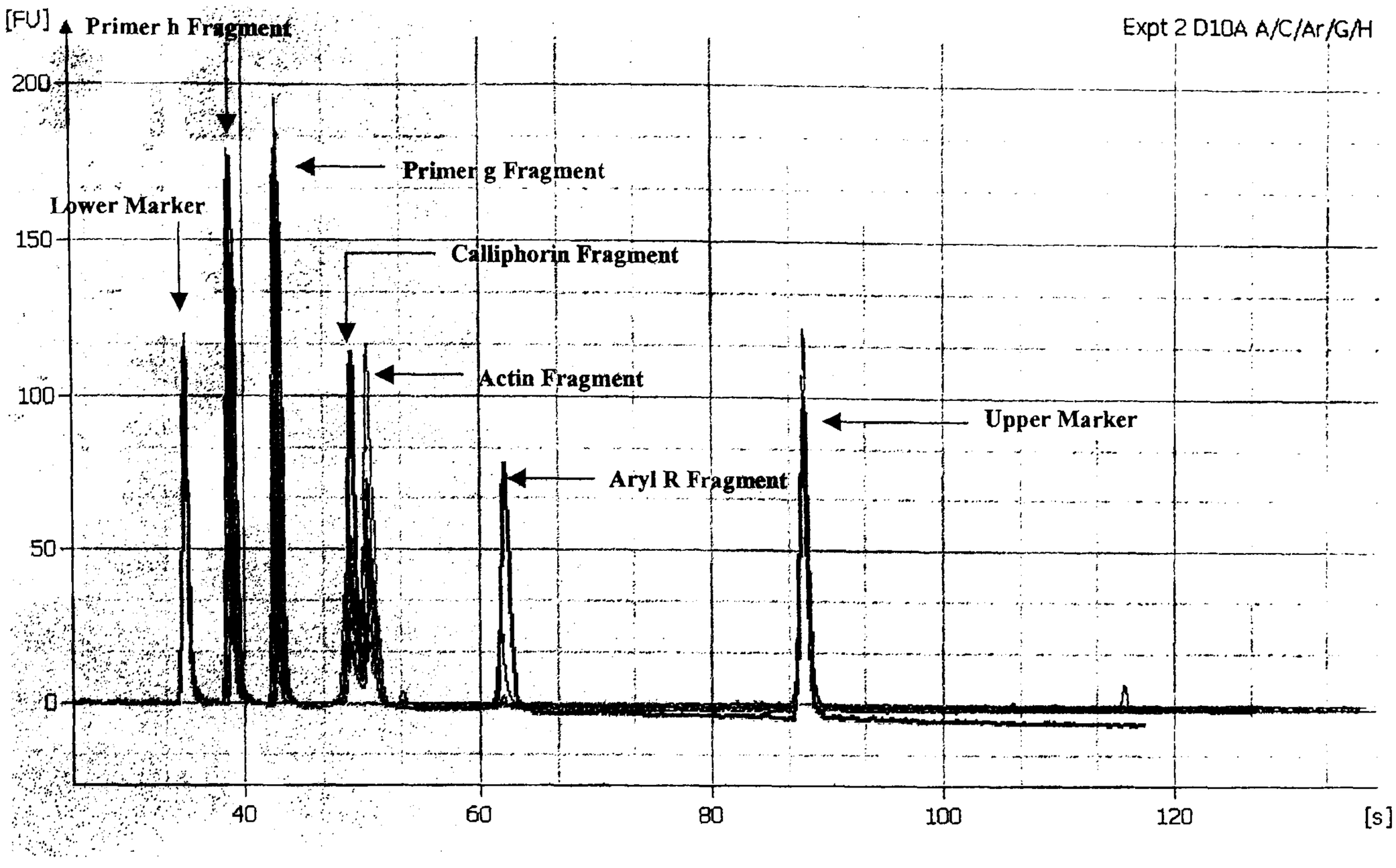

Figure 16.3. Overlay of pupal sample chromatograms of actin, calliphorin, primer g, primer h and arylphorin receptor (Aryl R) amplicons. The individual peaks are labelled according to the fragment they represent. The lower and upper markers allow comparison to the ladder (run separately, not shown) for sizing of amplicons. Markers are of known DNA concentration and thus comparison of peak area allows calculation of original concentrations of fragments.

Average values of PCR product for each gene and each timepoint were calculated along with the standard deviation. These values were then normalised against actin (Table 16.2). For some of the timepoints arylphorin receptor amplicons were not present (or at a low level to appear negative), this inhibits the use of the normalisation equation (as the numerator would be zero, thus necessitating $\log_{10}$ of zero). These values have been marked with an asterisk.

Table 16.2. Average PCR product concentrations±standard deviation of actin, Arylphorin R, Calliphorin, Primer g and Primer h for each pupal timepoint (Day 6 = third larval instar). The log_{10} of the ratio Potential marker gene/Actin is included for each timepoint. *not calculated

Day	ADH	Mean Concentration ± s.d. (ng/µl)					Log_{10} [Aryl R / Actin]	Log_{10} [Calliphorin / Actin]	Log_{10} [Primer g / Actin]	Log_{10} [Primer h / Actin]
		Actin	Arylphorin R	Calliphorin	Primer g	Primer h				
6(L3)	2880	8.77±0.77	1.02±1.39	3.24±0.89	11.60±8.19	10.31±2.44	-0.93	-0.43	0.12	0.07
7	3360	8.34±0.00	4.380±.00	4.74±0.00	7.01±0.00	13.21±0.00	-0.28	-0.25	-0.08	0.20
8	3840	4.08±2.15	1.40±1.52	4.75±1.59	9.98±4.31	11.25±5.28	-0.47	0.07	0.39	0.44
9	4320	4.04±0.59	1.44±1.67	3.03±0.65	7.42±5.27	11.32±1.76	-0.45	-0.13	0.26	0.45
10	4800	5.50±2.91	3.72±2.51	5.46±3.23	12.56±2.00	13.40±2.46	-0.17	0.00	0.36	0.39
11	5280	6.28±0.52	0.44±0.35	6.14±2.74	11.08±1.97	12.79±7.91	-1.15	-0.01	0.25	0.31
12	5760	2.72±1.08	0.70±1.10	3.92±1.41	7.16±2.42	11.30±2.74	-0.59	0.16	0.42	0.62
13	6240	3.70±1.92	0.93±1.10	3.82±0.82	10.43±4.43	11.18±3.70	-0.60	0.01	0.45	0.48
14	6720	4.26±0.67	0.20±0.28	5.45±0.44	13.04±2.94	13.73±4.08	-1.33	0.11	0.49	0.51
15	7200	5.25±0.70	0.15±0.25	8.29±4.26	17.34±7.97	34.23±19.12	-1.56	0.20	0.52	0.81
16	7680	5.76±0.37	0.98±0.67	3.23±0.34	13.05±3.25	12.40±3.58	-0.77	-0.25	0.36	0.33
17	8160	9.19±3.69	0.00±0.00	5.09±0.79	14.19±0.04	10.94±5.14	*	-0.26	0.19	0.08
18	8640	6.07±5.48	0.02±0.04	9.47±8.65	22.00±11.82	29.40±11.75	-2.54	0.19	0.56	0.69
19	9120	7.98±1.11	0.14±0.05	3.69±0.94	13.21±4.52	10.33±0.53	-1.77	-0.34	0.22	0.11
20	9600	2.62±2.93	0.00±0.00	5.96±6.82	36.16±1.09	48.85±5.30	*	0.36	1.14	1.27
21	10080	6.47±3.35	0.00±0.00	6.83±5.08	22.68±12.88	22.80±10.62	*	0.02	0.55	0.55
22	10560	9.47±1.85	0.14±0.19	4.52±1.13	15.99±0.25	13.50±2.21	-1.85	-0.32	0.23	0.15
23	11040	3.97±3.17	0.16±0.22	5.48±0.75	17.50±6.17	21.36±8.72	-1.41	0.14	0.64	0.73

The log_{10} [Potential marker gene/actin] values were then plotted against ADH for the calliphorin, primer g and primer h gene products (Figures 16.4, 16.5 and 16.6).

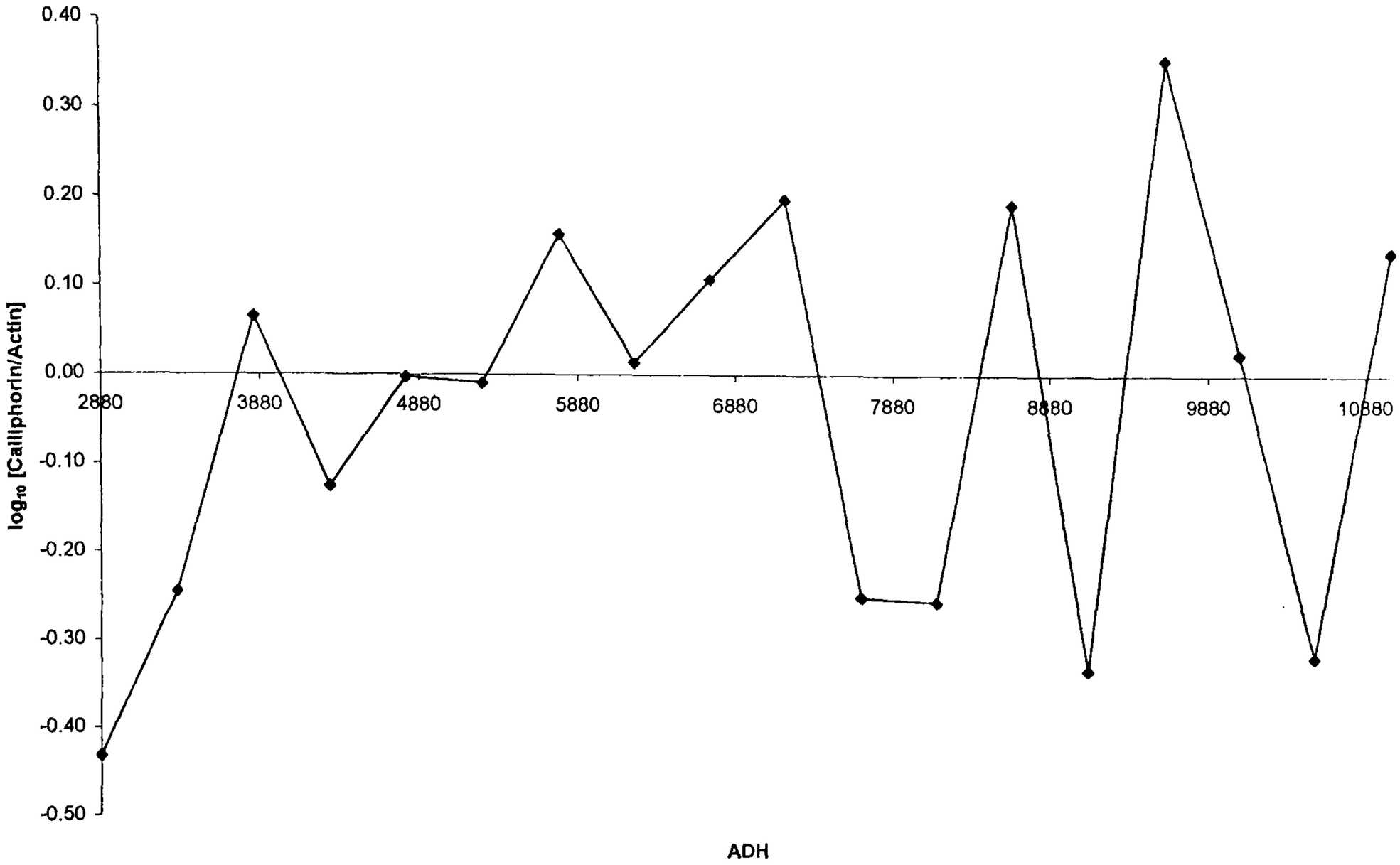

Figure 16.4. Log_{10} [calliphorin / actin] plotted against ADH for the pupal stage of *C.vicina*.

The initial amount of calliphorin is very low in the larval three sample (2880 ADH) and increases in expression during the pupal stage. During the end of the pupal stage the calliphorin levels rise and fall dramatically. Due to the alternating expression levels during the end of the pupal stage this gene would not provide useful information as to the age of an unknown *C. vicina* pupal sample.

The primer g and primer h *C. vicina* pupal expression profiles are similar during the pupal stage. Both have relatively low expression at the beginning of the pupal stage. Both have a distinct peak at 9600 ADH, which could be used as a marker for this timepoint during the pupal phase.

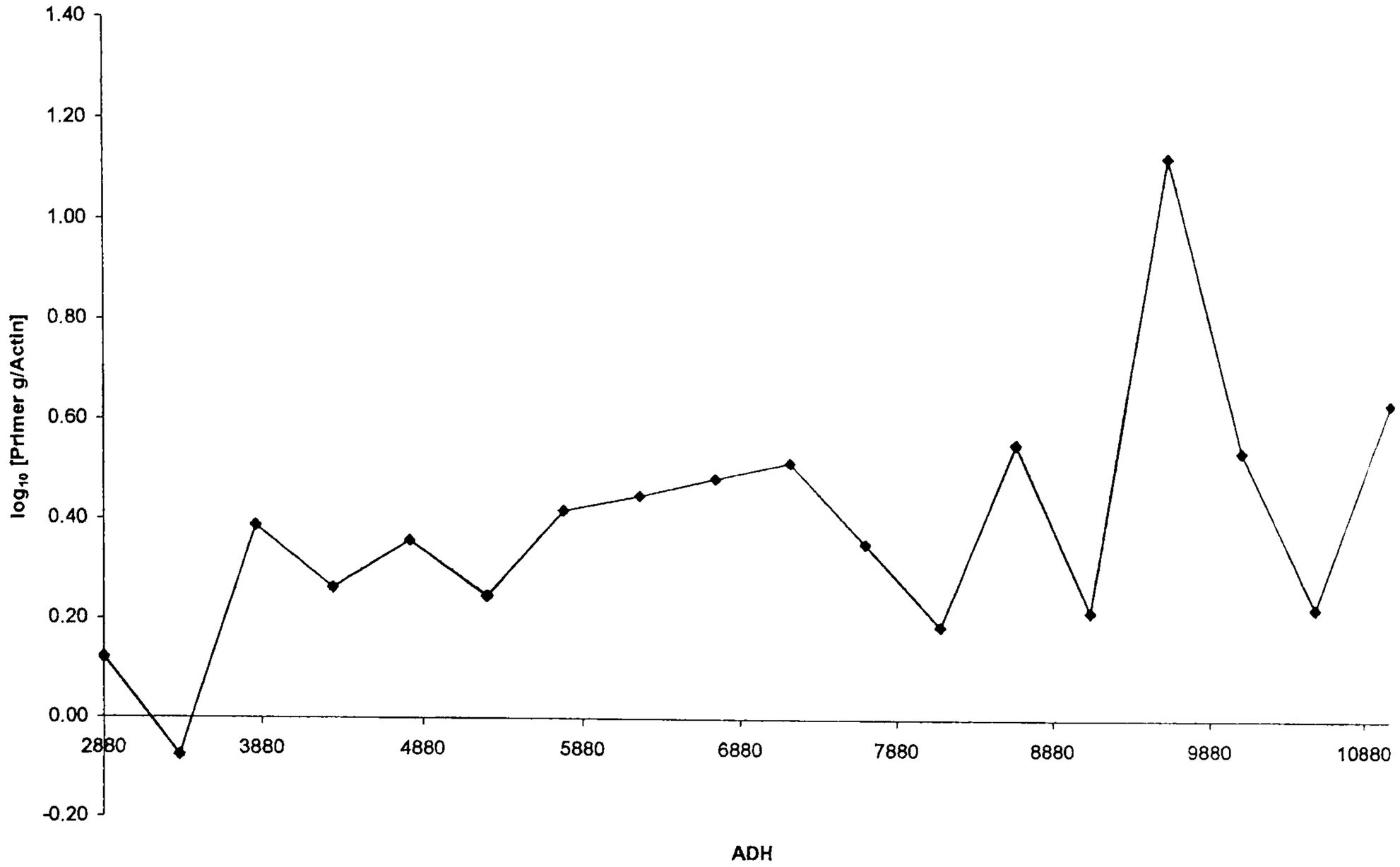

Figure 16.5. Log_{10} [primer g / actin] plotted against ADH for the pupal stage of *C.vicina*.

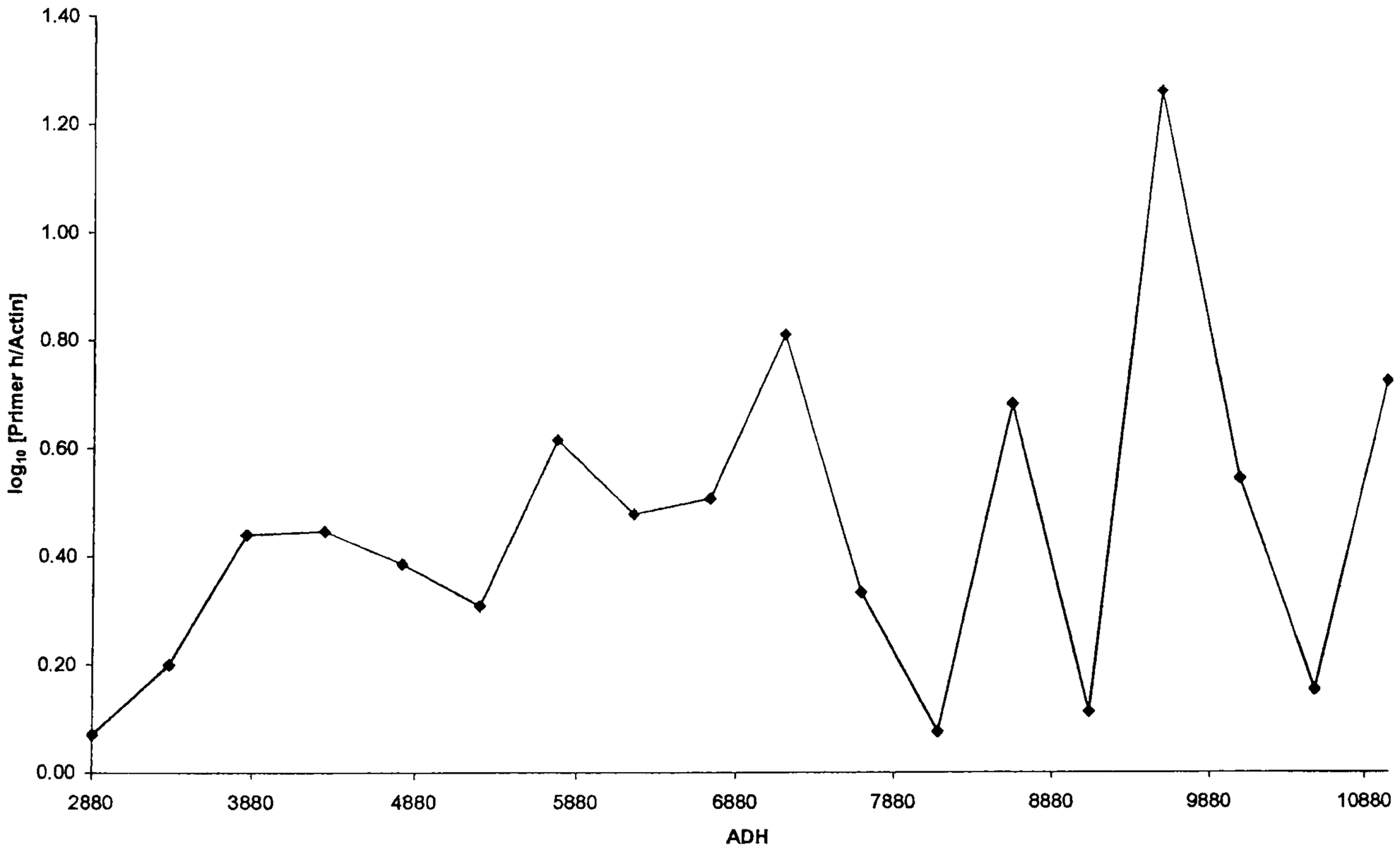

Figure 16.6. Log_{10} [primer h / actin] plotted against ADH for the pupal stage of *C.vicina*.

As some of the values for arylphorin receptor could not be normalised against the actin quantities the separate actin and arylphorin receptor concentrations were plotted (Figure 16.7).

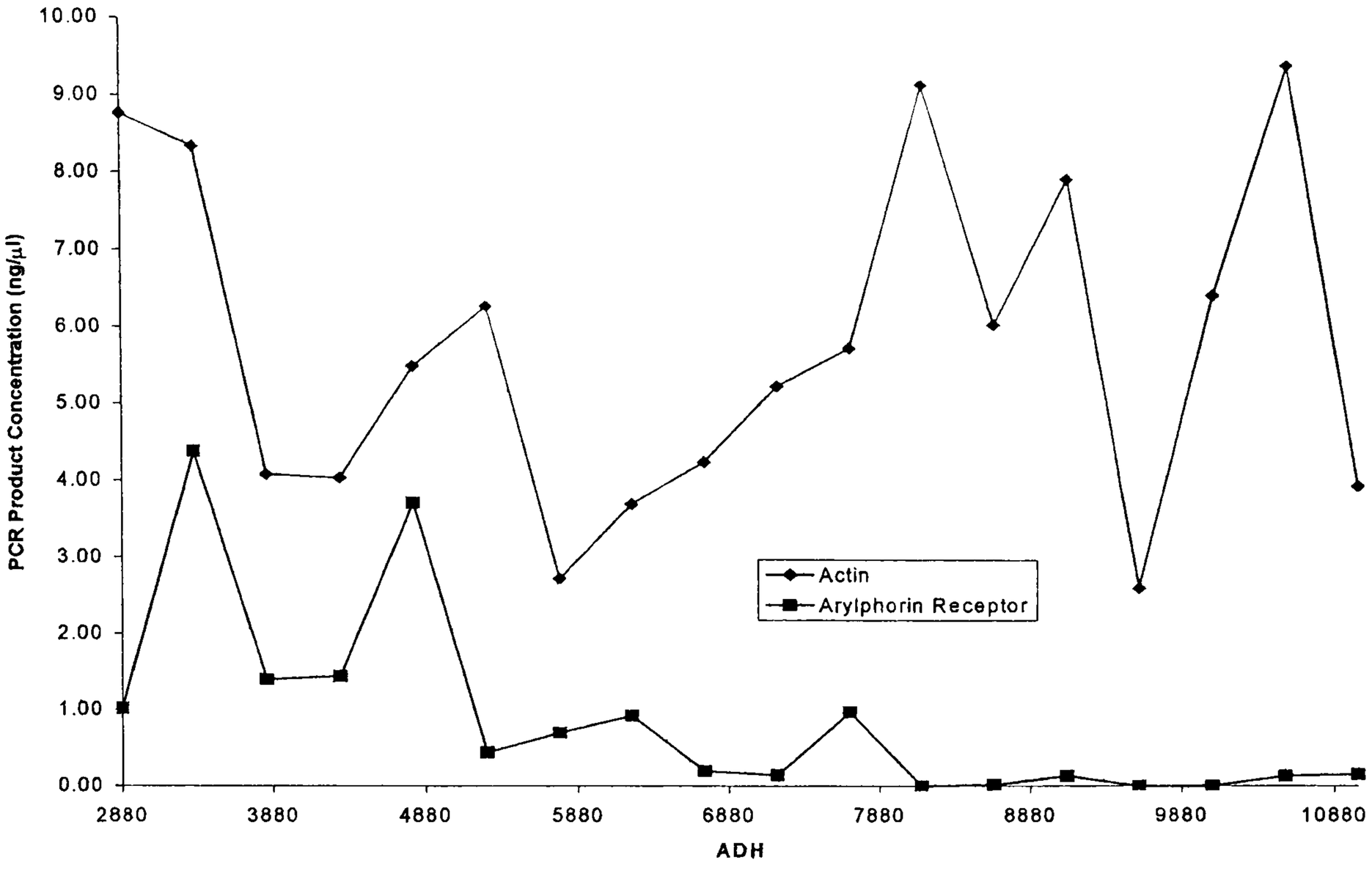

Figure 16.7. Amplicon concentration for arylphorin receptor and actin genes plotted against various *C. vicina* pupal timepoints.

The graph illustrates that in the later pupal stage the actin levels are relatively high whereas the levels of arylphorin receptor are around zero. Arylphorin receptor expression does appear to be occurring at the beginning of the pupal stage and to examine this further the normalised values for timepoints 2880 – 7680 ADH were calculated. These $\log_{10}$ [arylphorin receptor/actin] values were then plotted against ADH (Figure 16.8).

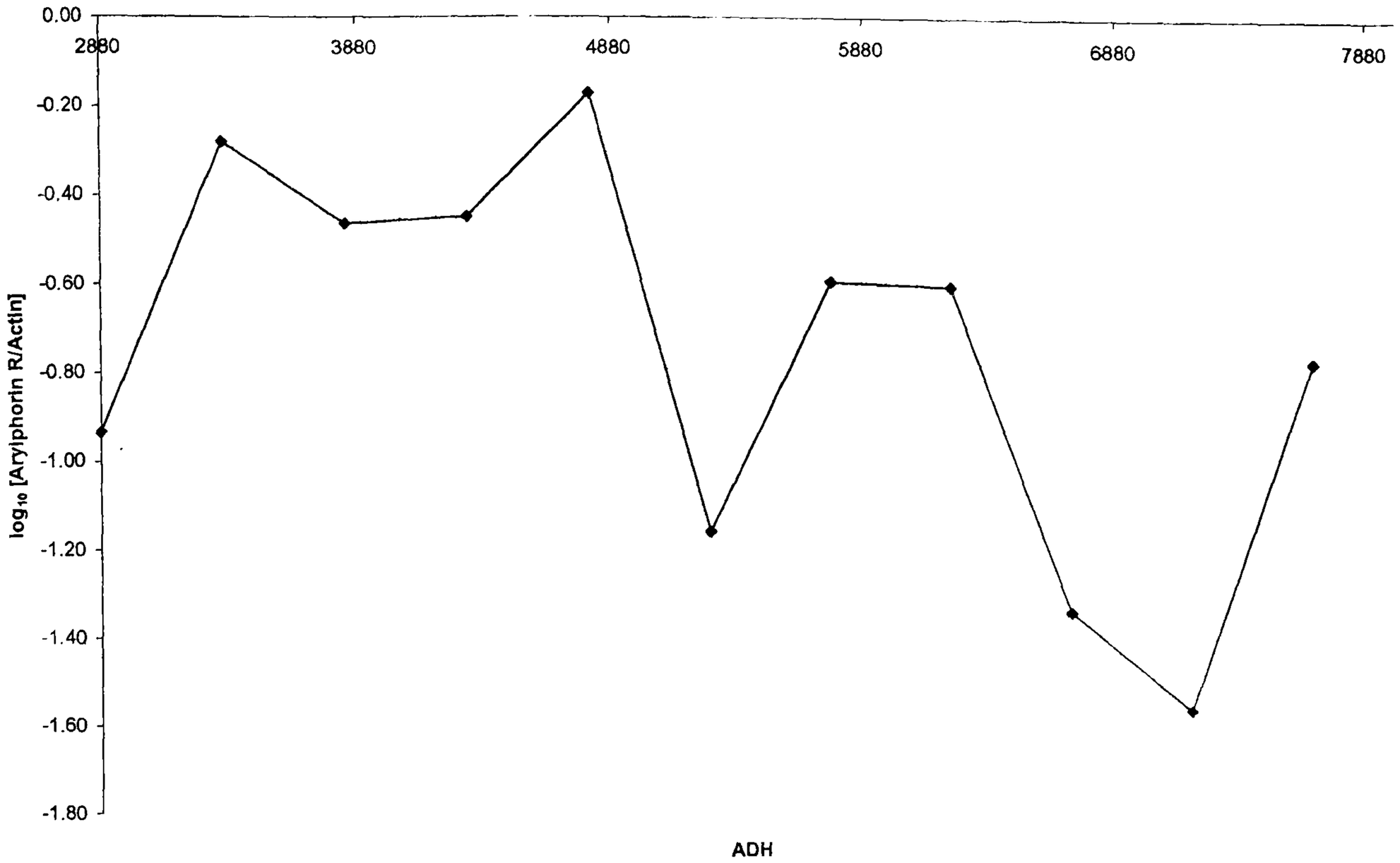

Figure 16.8. Log_{10} [arylphorin receptor / actin] plotted against ADH for the early pupal stage of *C. vicina*.

This graph illustrates an initial rise after the larval stage followed by a steady decline of the level of arylphorin receptor. Combination of Figures 16.7 and 16.8 implies that this steady decline at the end of the *C. vicina* pupal stage after 7680 ADH continues until no further arylphorin receptor expression occurs. The levels of arylphorin receptor gene expression fall to zero and thus can provide an indication of early/late pupal stage.

From these preliminary findings it appears that LSP-2, primer g/primer h and arylphorin receptor might be used as molecular markers to help in determination of pupal age. Calliphorin does not appear to be useful in age determination.

However there are established problems with using endpoint quantification as a measure of original DNA quantity, and these are discussed in the following section.

16.3 Problems with Endpoint Quantification of PCR Products

One problem with using agarose gels or capillary electrophoresis to quantify PCR products is that products are only differentiated upon size. Amplicons of different DNA regions cannot be of similar length, as they will not appear as different products. The amplicons can also neither be too long, as they will be out of the range of the capillary electrophoresis kits or too short for the same reason and also as there will be poor resolution on agarose gels.

However, the main problem with quantifying at the end of amplification is due to the amplification process itself. The PCR is made up of three phases (Figure 16.9). In theory amplification should be exponential. The numbers of product fragments should double with each cycle. This is demonstrated in the initial exponential phase of the PCR. However as the PCR continues, reagents get consumed and this changes the rate of reaction so that products are not doubled each cycle. This rate of change is not a constant from run to run. This is called the linear phase of the reaction. Eventually reactions slow down completely and amplification reaches a plateau – increases in cycle number will not increase the amount of DNA. It is after this phase that conventional agarose gel detection will take place. Due to a non-consistent change in rate after the exponential phase two samples with the same starting amount of DNA will show differences in final amounts of amplified target.

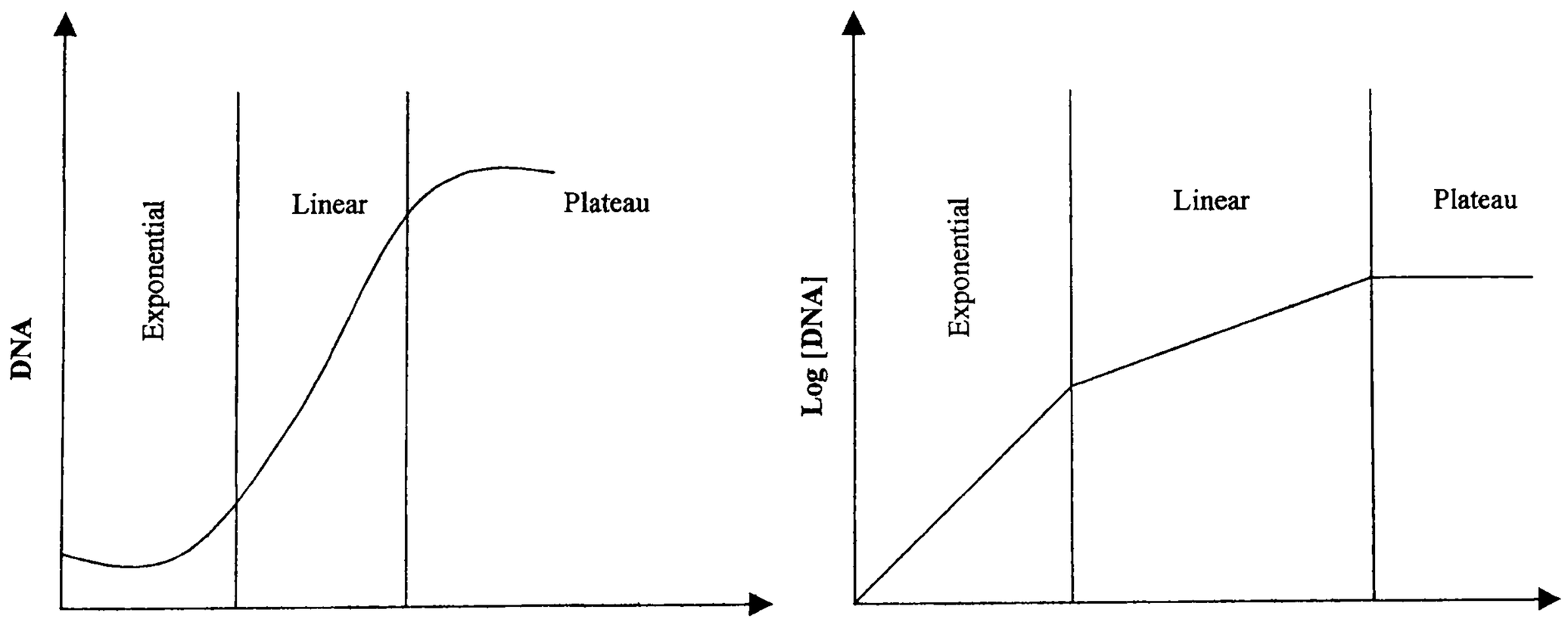

Figure 16.9. The three phases of a PCR reaction demonstrated using amount of DNA against cycle number in the left hand graph and log [DNA] versus cycle number in the graph on the right.

An alternative to endpoint quantification would be to use real time PCR.

Chapter 17

Quantification of gene expression II

Real Time PCR Quantification

As illustrated in Figure 16.9, the PCR occurs in three phases. In a real time PCR quantification is measured during the exponential phase of the reaction, where reagents are non-limiting and therefore amplicons are doubling each cycle.

The PCR set-up is essentially the same as traditional PCR except that the real time PCR contains fluorescent molecules whose signal increases in proportion to the amount of PCR product. The use of fluorescence as a detection method whilst the amplification is occurring eliminates the need for separation of amplicons by electrophoresis. This has three benefits, one is that different gene products need not be different lengths, the second is the lack of post-PCR processing which means the quantification method is more time efficient and lastly the use of fluorescence is a more sensitive technique thus allowing detection of low level gene expression.

The simplest of the florescent molecules available is the intercalating dye, SYBR Green I. This dye has low fluorescence when unbound but as DNA fragments are elongated the dye becomes incorporated into the double stranded molecules and fluoresces when excited by a specific wavelength emitted from a laser. This method is non-specific – the dye binds to any double stranded DNA.

A more specific method is to use probes complementary to the target DNA. This was used in this work. QuantiProbes are custom made probes designed by QIAGEN software. These probes are specific to the target sequence and include a fluorophore, a non-fluorescent quencher and minor groove binder (MGB). The proximity of the quencher to the fluorophore ensures that there is no fluorescence emitted when the probe is in solution. On binding to its target sequence, the conformation of the probe changes and the quencher molecule is physically separated from the fluorophore, thus creating a signal This signal is proportional to the amount of PCR product present. Thus quantification of target sequence can be established. The MGB prevents the hydrolysis of the probe by *Taq* polymerase. The probes are displaced during the extension step of PCR to allow amplification of the target sequence. For this reason fluorescence readings are taken during the annealing phase of the PCR when the

probes are bound to the DNA. The probes are designed along with primers for the target genes, so that they are non-complementary and will not form primer-primer or primer-probe duplexes.

It was unfeasible to amplify all the gene products from Chapter 16 due to the great difference in cost between the two types of quantification. Arylphorin receptor, LSP-2 and primer g were chosen for real time PCR analysis along with the housekeeping actin for normalisation. Calliphorin was not included, as from the preliminary work, it did not vary significantly in expression during the pupal stage. Primer h appears to have a similar gene expression pattern to primer g and thus was not included.

The GenBank sequences used in Chapter 14 for design of primers were used again to design real time PCR primers and probes. Primers needed to be redesigned, as it is best practice in real time PCR to amplify regions of around 100bp. Also, QIAGEN design the primers to be compatible with their other reagents and probes and so that they have comparable annealing temperatures, to guarantee experimental success.

Before the primers and probes can be used for cDNA quantification, their amplification efficiency first needs to be assessed. As gene quantifications for each sample will be compared to those of actin, the primers and probes for each gene product needs to be examined to make sure they are working with similar efficiency. Any differences in final DNA quantities can therefore be concluded as differences in starting amounts of mRNA as opposed to the real time PCR working with different efficiencies.

17.1 Materials and Methods

17.1.1 Samples

This real time PCR work utilised the cDNA samples synthesised from RNA extracted from the first experimental sampling of pupae (Chapter 12).

17.1.2 Primer and Probe Design

For this real time PCR quantification a QuantiTect® Custom Assay (QIAGEN) would be used for each gene product. The following primers and dye labelled probes were

designed using QIAGEN online software and are presented in Table 17.1 (https://customassays.qiagen.com/design/inputsequences.asp).

Table 17.1. Primers and QuantiProbes for real time PCR. YY™ = Yakima Yellow™. FAM and YY are dye labels.

Gene Product	Primer/Probe	Sequence (5'-3')	Dye label
actin	Forward primer	TCAAGTCATCACCATCGGTAA	n/a
	Reverse primer	ACCGCAAGATTCCATACCCAA	n/a
	QuantiProbe	CCCTCTTCCAACCCTCA	FAM
primer g	Forward primer	CGCAAAGCCCAACAAGAA	n/a
	Reverse primer	TTGGCACGGAATTTGCTGATG	n/a
	QuantiProbe	GAAGAAGCCGAAGAAC	YY™
LSP-2	Forward primer	GCGAAATTCCACACTTCAACA	n/a
	Reverse primer	GCGAGCACCATAGTATTCGAT	n/a
	QuantiProbe	ACCAACCCTACGAACA	YY™
arylphorin receptor	Forward primer	CAGACAATGCAGGGTATAAGAG	n/a
	Reverse primer	GGGGCATATAGACCTGATGTAA	n/a
	QuantiProbe	GGAGTTGGTGGTGGTA	YY™

17.1.3 Thermal cycling

The real time PCR amplifications were carried out according to manufacturer's instructions for the QuantiTect® Custom Assay kit. Reactions were set up in 96 well plates (Applied Biosystems) and these were covered using optical adhesive covers (Applied Biosystems).

All reactions contained 12.5µl of 2X QuantiTect® Probe PCR Master Mix, 2.5µl of 10X Primer Mix containing both forward and reverse primers, 2.5µl of 10X QuantiProbe solution, 2µl template cDNA and 5.5µl ddH_2O. The following two sections (Sections 17.1.4 and 17.1.5) explain exactly which samples were run.

The plates were run on the ABI Prism 7000 (Applied Biosystems) using the following thermal program. Initial *Taq* polymerase activation step of 95°C for 15 min followed by 40 or 45 cycles of 76°C for 30s, 94°C for 15s and 56°C for 30s. The cycling phase

begins with the extension stage so that the annealing stage and the fluorescence readings occur in the last stage of the cycle.

17.1.4 Primer and Probe efficiency testing

To test the efficiency of each set of primers and probes, a series of dilutions of pooled cDNA from all the sample timepoints were amplified with conditions as described in Section 17.1.3 with thermal cycling for 40 cycles. The dilution series was neat, 1 in 10, 1 in 100, 1 in 1000, 1 in 10,000 and ddH_2O as a negative control. Each dilution for each gene product was duplicated on the plate.

All genes including actin were initially run on one plate. However on examination of the LSP-2 data (the amplification plots were poor) and after consultation with QIAGEN technical support, the LSP-2 samples were repeated in a run of 45 cycles along with actin for comparison.

17.1.5 Pupal Samples

As there was not the capacity on one 96 well plate to run all samples for all genes concurrently, three plates were run. All were set up with the quantities of reagents as mentioned in Section 17.1.3. The first plate contained timepoints 2880, 3360, 3840, 4320 and 4800 ADH. The second contained timepoints 5760, 6240, 6720, 7200 and 7680 ADH. The third contained timepoints 8640, 9120, 9600, 10080 and 10560 ADH. For each timepoint both replicates from the cDNA synthesis were included along with the original RNA sample (negative sample control). The plate also contained duplicate negative reagent controls (ddH_2O in place of template) for each gene amplification set. Each plate contained reactions for the four different gene products including the actin primers/probe. This was to avoid any differences due to plate-to-plate variation. Thermal cycling took place as discussed in Section 17.1.3 using 45 cycles.

17.1.6 Data Analysis

17.1.6.1 Setting the Threshold and Baseline

Before data can be analysed the threshold and baseline need to be examined on the amplification plot. Background fluorescence naturally exists in the plate wells and thus there is a certain level of background noise in each run. This background noise is most evident during the initial cycles of PCR, when significant amounts of

amplification products have not yet accumulated. The software automatically sets the background as occurring before 15 cycles but if the fluorescence emerged above the baseline before the fifteenth cycle this was altered.

As mentioned previously the point of quantification during RT-PCR is in the exponential phase as this is where quantification will be most accurate. The software on the ABI PRISM 7000 allows the user to manipulate the point (or threshold) at which quantification occurs to increase accuracy. The amplification plot was changed so that the florescence values are plotted on a log scale. The exponential phase appears linear in this view and the threshold was placed so it is in the exponential phase of all plots. Once the threshold has been set, the threshold cycle values (CT) for each sample were read from the graph. These reflect the cycle number at which the fluorescence level exceeds the threshold and form the raw data from which quantification and relative quantification can be calculated. The greater the original copy number of the target, the sooner the threshold will be crossed and thus the lower the CT value.

17.1.6.2 Primer and Probe Efficiency

Standard curves for each of the gene products were produced using Microsoft Excel®. These are plots of the log of the dilution against CT values. The slope the regression line should be close to –3.32 (calculated from $10^{[-1/slope]} = 2$). Efficiency (%) therefore is equivalent to $50 \times 10^{[-1/slope]}$.

Correlation coefficients for closeness of fit of the replicates to the line are also given. These curves can also be used as a method for assessing whether the threshold has been put in the most accurate position.

To assess whether the primer/probes are working at the same efficiency the differences between the CT values for each gene and actin for each dilution in the series was plotted against the log of the dilution factor of the pooled cDNA sample. This was done in Microsoft Excel®.

17.1.6.3 Comparative CT Method ($2^{-\Delta\Delta CT}$)

This arithmetic method of comparing gene expression values is based upon the equation that describes the exponential amplification of PCR (ABI User Bulletin 2, Livak and Schmittgen 2001).

$$X_T = X_0 \times (1 + E_X)^{CT}$$

X_T – number of molecules at threshold

X_0 – number of molecules initially

E_X – efficiency of target amplification

^{CT} – threshold cycle number

Rearrangement of this equation, assuming that efficiencies for the PCR reactions are equal, gives the following equation for the relative quantification of a target sample normalised to the actin housekeeping gene and relative to another sample (calibrator).

$$X = 2^{-\Delta\Delta CT}$$

Where, $\Delta\Delta CT = \Delta CT$ target - ΔCT calibrator.

ΔCT target = CT target – CT reference

The standard deviation for these values can be calculated by using the following equation.

Standard deviation of ΔCT,

$$s = \sqrt{s_{target}^2 + s_{reference}^2}$$

This value also applies for $\Delta\Delta CT$

The range of 'X' is determined by evaluating $2^{-\Delta\Delta CT}$ with $\Delta\Delta CT \pm s$

17.2 Results

17.2.1 Primer and Probe Efficiency

The amplification plots are found in Appendix X. Once the baseline had been determined as described in Section 17.1.6.1, the CT values for each of the amplification products was evident. Raw CT data is presented in Table 17.2.

Table 17.2. CT values for actin, arylphorin receptor and primer g dilutions. Undetermined values are those where the amplification plot has not risen sufficiently to cross the threshold.

	actin		arylphorin receptor		primer g	
	CT	Mean±sd	CT	Mean±sd	CT	Mean±sd
Neat 1	19.30	18.515 ±1.114	25.98	25.670 ±0.436	26.03	26.775 ±1.054
Neat 2	17.73		25.36		27.52	
1 in 10 1	22.77	22.290 ±0.677	28.64	28.405 ±0.333	29.46	29.470 ±0.016
1 in 10 2	21.81		28.17		29.48	
1 in 100 1	23.49	24.375 ±1.256	31.92	31.690 ±0.322	32.71	32.955 ±0.342
1 in 100 2	25.26		31.46		33.20	
1 in 1000 1	28.22	28.985 ±1.047	35.34	35.230 ±0.15	35.70	37.105 ±1.993
1 in 1000 2	29.71		35.12		38.51	
1 in 10000 1	30.02	30.820 ±1.134	38.67	38.455 ±0.304	Undetermined	
1 in 10000 2	31.62		38.24		Undetermined	
Water 1	37.90	36.380 ±2.146	Undetermined		Undetermined	
Water 2	34.86		Undetermined		Undetermined	

Table 17.3. CT values for actin and LSP-2 dilutions. Undetermined values are those where the amplification plot has not risen sufficiently to cross the threshold.

	actin		LSP-2	
	CT	Mean±sd	CT	Mean±sd
Neat 1	18.92	19.150	20.87	20.555
Neat 2	19.38	±0.321	20.24	±0.443
1 in 10 1	22.17	22.095	24.66	24.350
1 in 10 2	22.02	±0.106	24.04	±0.442
1 in 100 1	25.56	25.785	28.01	28.110
1 in 100 2	26.01	±0.319	28.21	±0.140
1 in 1000 1	29.11	29.105	32.45	32.310
1 in 1000 2	29.10	±0.012	32.17	±0.199
1 in 10000 1	32.83	32.945	37.95	37.790
1 in 10000 2	33.06	±0.163	37.59	±0.259
Water 1	35.33	35.330	Undetermined	

Standard curves of CT versus log [dilution] for each primer/probe set are illustrated in Figures 17.1-4.

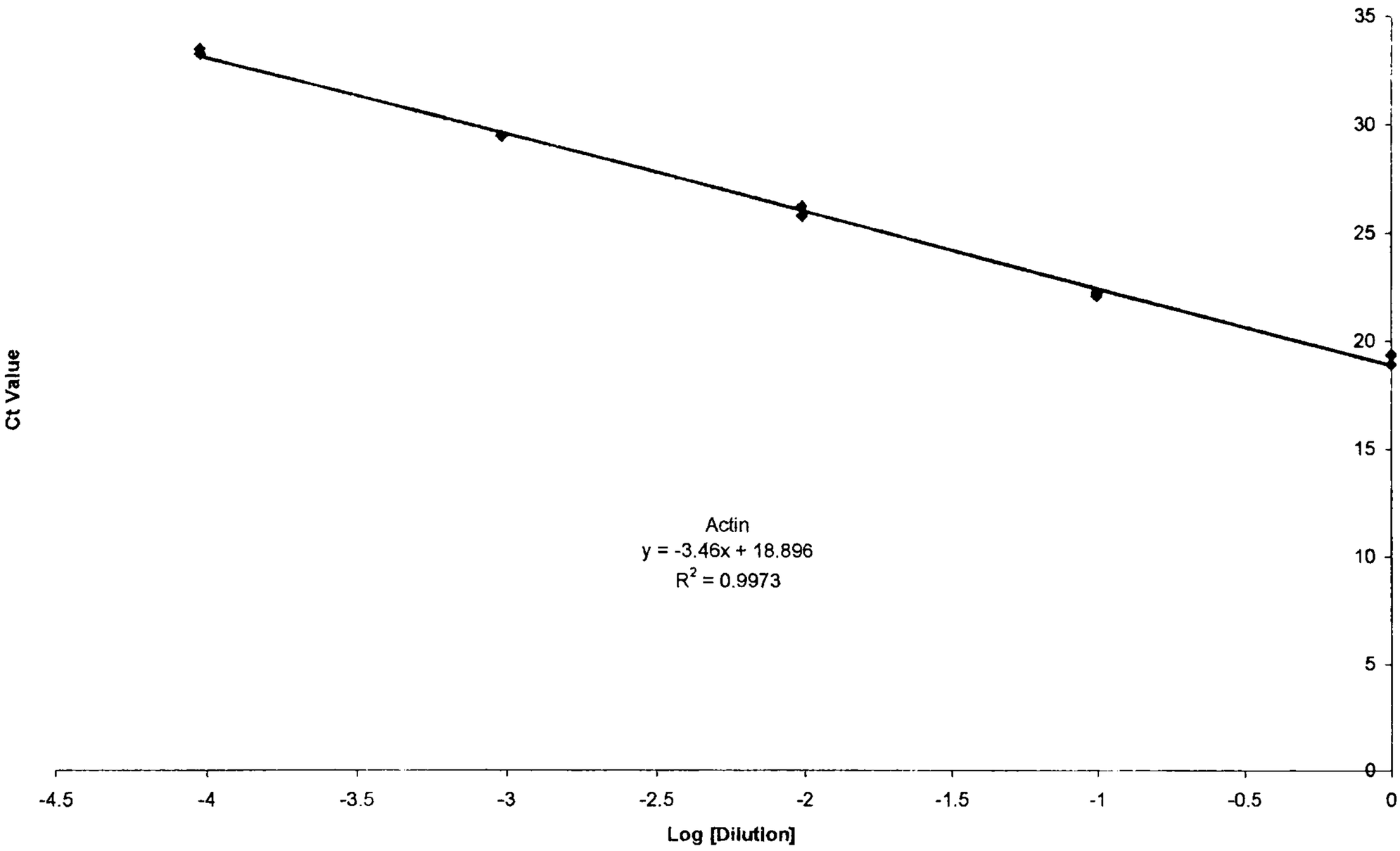

Figure 17.1. Actin real time PCR amplicon CT values plotted for each of the dilutions of cDNA.

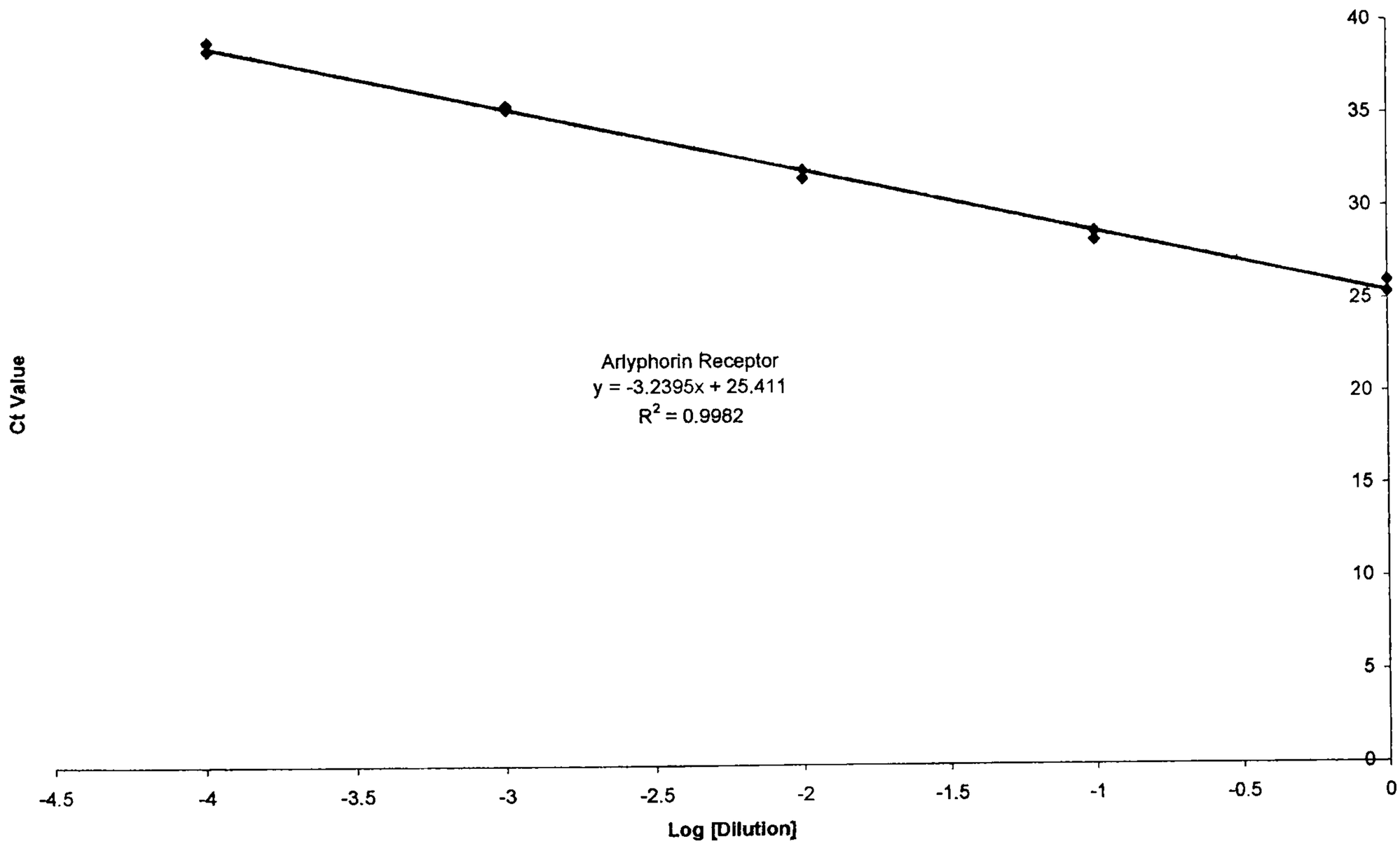

Figure 17.2. Arylphorin receptor real time PCR amplicon CT values plotted for each of the dilutions of cDNA.

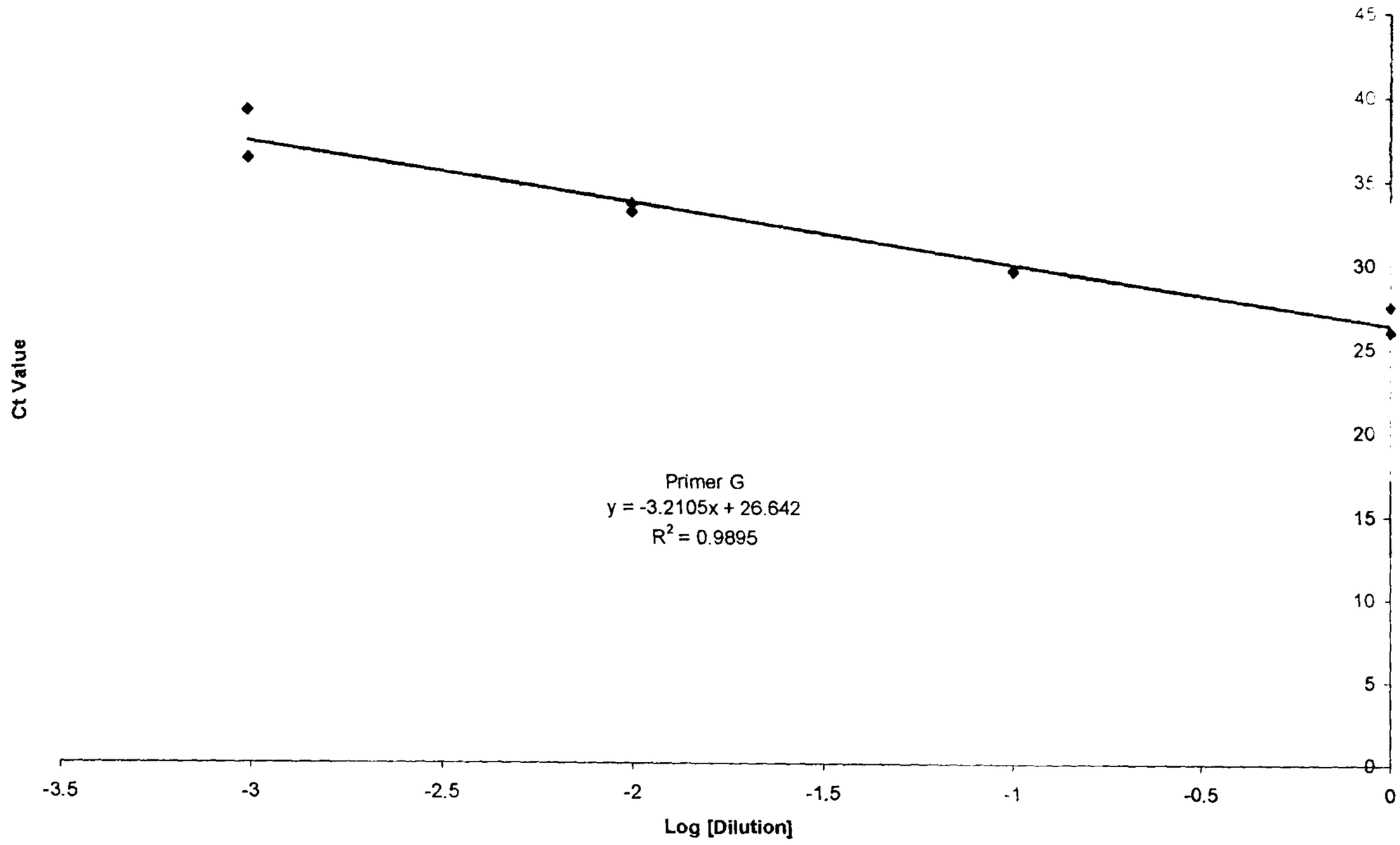

Figure 17.3. Primer g real time PCR amplicon CT values plotted for each of the dilutions of cDNA.

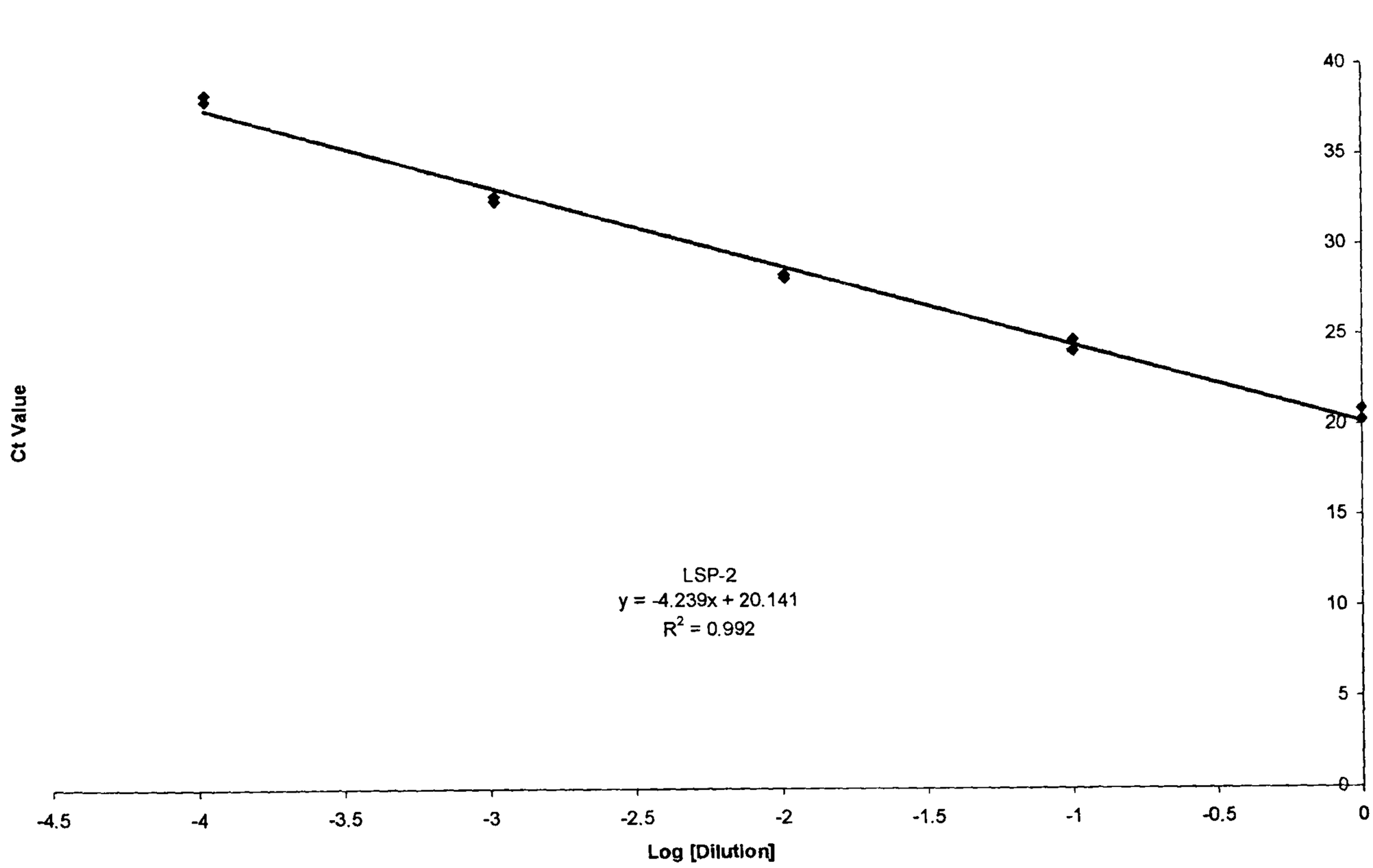

Figure 17.4. LSP-2 real time PCR amplicon CT values plotted for each of the dilutions of cDNA.

The slopes for the regression lines for each gene product are –3.46, -3.24, -3.21 and –4.24 for actin, arylphorin receptor, primer g and LSP-2 respectively. This produces efficiencies of 97.3%, 101.7%, 102.4% and 86% for the primer and probe sets.

The CT values for each gene product were then compared to the actin data to assess similarities between primer/probe efficiencies. The regression lines are present in Figures 17.5 and 17.6.

If the primers are of comparable efficiency they will amplify each dilution similarly. The difference between the CT values for the gene and the housekeeping gene should not vary between dilutions (and thus samples in later experiments). Therefore the slope of the regression line plotted on the graph should be approximately zero.

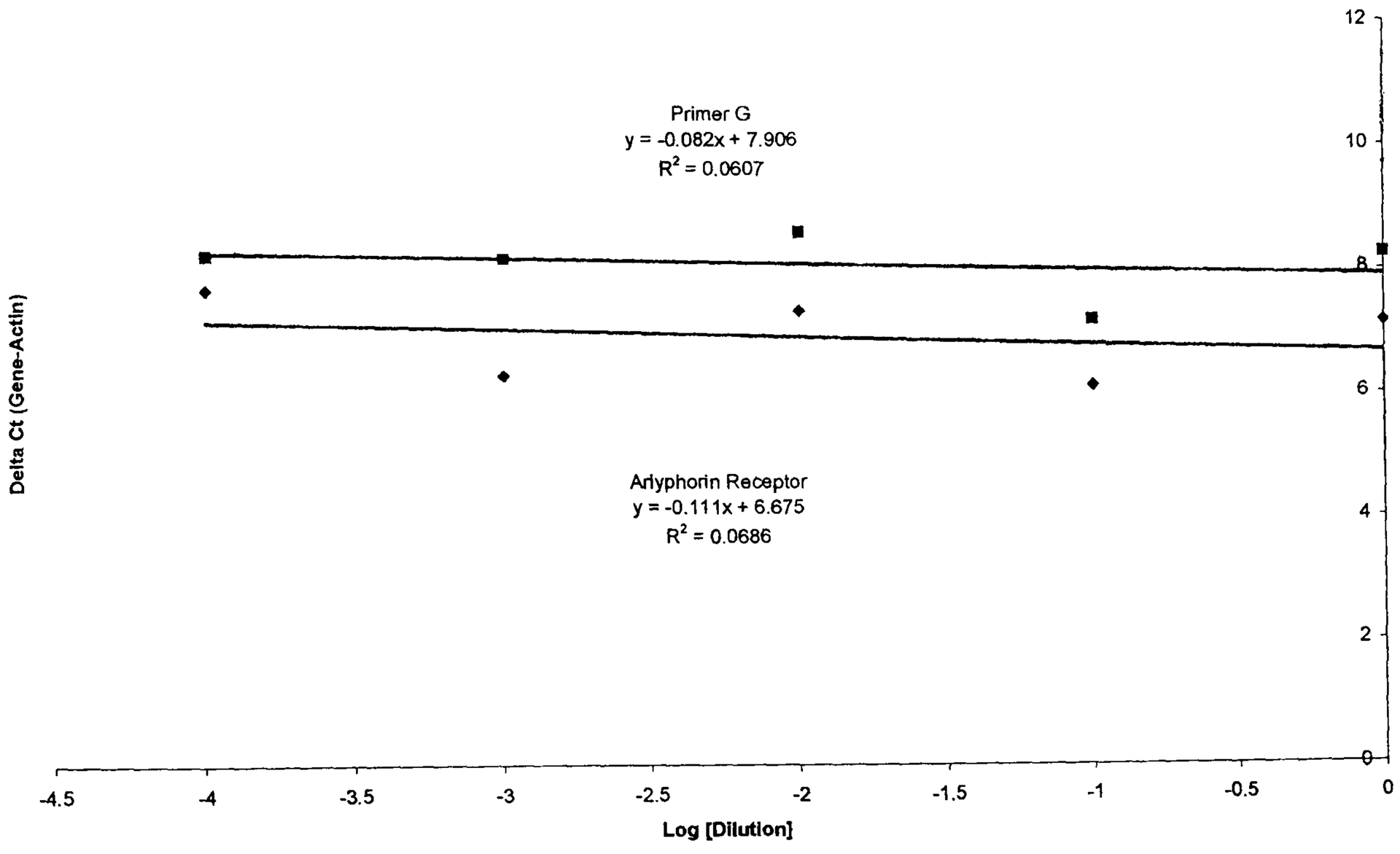

Figure 17.5. Arylphorin receptor and primer g CT values for the pupal phase normalised against actin for a cDNA dilution series.

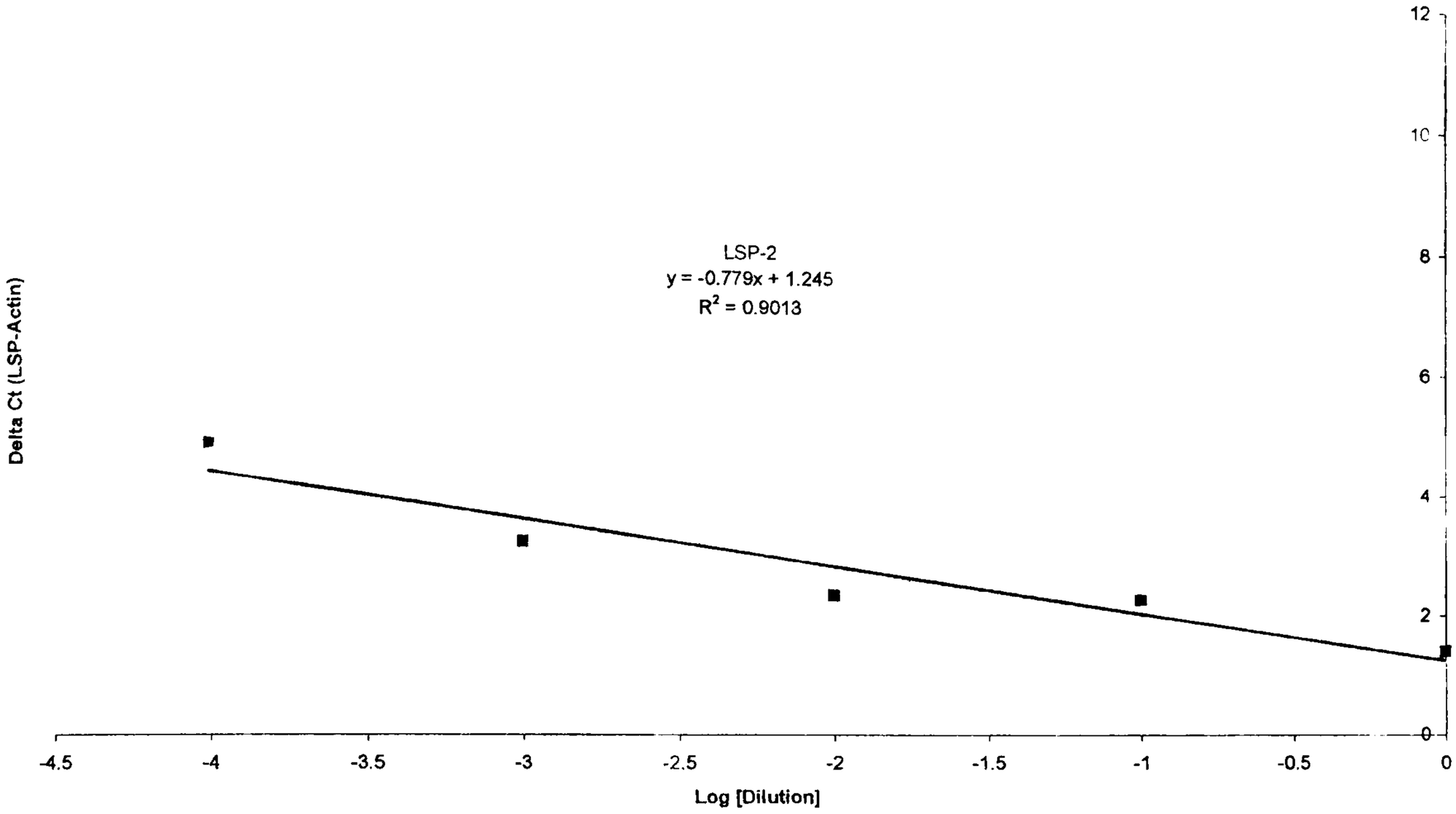

Figure 17.6. LSP-2 CT values for the pupal phase normalised against actin for a cDNA dilution series.

The slopes of the regression lines are -0.08, -0.11 and -0.78 for arylphorin receptor, primer g and LSP-2 respectively. This indicates that the actin, arylphorin receptor and primer g primers/probes are working with similar efficiencies. The LSP-2 primers/probe do not appear to have a constant efficiency over the dilution series when compared to the actin RT-PCR, the slope of the regression line is greater than 0.7. The LSP-2 data for the actual samples will have to be analysed differently, it cannot be compared to the actin CT values. Instead this data will be compared to the CT values for the dilution series, as recommended by the Applied Biosystems (User Bulletin 2).

17.2.2 Pupal Timepoints

17.2.2.1 Arylphorin receptor and primer g

Amplification plots from the RT-PCR runs can be found in Appendix XI along with raw CT data. The average CT values were calculated for each of the pupal timepoints for each gene product including actin; these are presented in Table 17.4. The average CT values for arylphorin receptor and primer g were normalised by subtracting the CT values for actin (ΔCT). Day 6 (2880ADH) ΔCT was subtracted from all other ΔCT values as described in Section 17.1.6.3. The final figure of $2^{-\Delta\Delta CT}$ indicates the pupal gene expression relative to the end of the larval phase (Day 6; 2880ADH).

Table 17.4. Normalised CT values for Arylphorin receptor (Aryl R) and Primer g with Actin expression compared with Day 6 for *C. vicina* pupae.

Day	ADH	Average CT			ΔCT		ΔΔCT		$2^{-\Delta\Delta CT}$ Relative to Day 6	
					$CT_{Aryl\ R} - CT_{Actin}$	$CT_{Primer\ g} - CT_{Actin}$	ΔCT - ΔCT (Day6)			
		Actin	Aryl R	Primer g			Aryl R	Primer g	Aryl R	Primer g
6	2880	16.86±0.62	21.20±0.07	23.91±0.31	4.34±0.62	7.06±0.69	0.00±0.62	0.00±0.69	1.00 (0.6-1.5)	1.00 (0.6-1.6)
7	3360	17.42±0.20	19.07±0.60	24.25±0.68	1.65±0.63	6.83±0.71	-2.69±0.63	-0.23±0.71	6.45 (4.2-10.0)	1.17 (0.7-1.9)
8	3840	19.24±0.04	19.36±0.31	25.31±0.29	0.13±0.31	6.07±0.29	-4.22±0.31	-0.99±0.29	18.57(15.0-23.1)	1.98 (1.6-2.4)
9	4320	21.75±0.54	17.14±0.35	26.53±0.70	-4.62±0.64	4.78±0.88	-8.96±0.64	-2.28±0.88	496.28(319-777)	4.86 (2.6-9.0)
10	4800	22.73±0.43	21.37±1.51	28.15±0.01	-1.37±1.57	5.42±0.43	-5.71±1.57	-1.64±0.43	52.16 (17.6-155)	3.12 (2.3-4.2)
12	5760	23.08±0.02	21.49±0.08	28.15±0.26	-1.59±0.09	5.07±0.26	-5.93±0.09	-1.99±0.26	60.97 (57.4-64.7)	3.97 (3.3-4.8)
13	6240	21.00±0.17	20.93±0.35	25.06±0.35	-0.07±0.39	4.07±0.39	-4.41±0.39	-2.99±0.39	21.19 (16.2-27.8)	7.94 (6.1-10.4)
14	6720	20.91±0.45	22.34±0.74	25.93±0.68	1.43±0.87	5.03±0.81	-2.91±0.87	-2.03±0.81	7.52 (4.1-13.7)	4.08 (2.3-7.2)
15	7200	28.08±1.06	30.88±0.58	32.58±0.03	2.81±1.21	4.50±1.06	-1.54±1.21	-2.56±1.06	2.90 (1.3-6.7)	5.88 (2.8-12.3)
16	7680	19.35±0.23	19.69±0.08	23.11±0.04	0.34±0.25	3.76±0.24	-4.00±0.25	-3.30±0.24	16.00 (13.5-19)	9.82 (8.4-11.6)
18	8640	28.90±0.95	28.20±0.99	30.47±0.61	-0.70±1.38	1.58±1.13	-5.04±1.38	-5.48±1.13	32.90 (12.7-85.4)	44.63 (20.3-97.9)
19	9120	19.66±0.16	21.80±0.72	19.23±0.27	2.14±0.74	-0.43±0.31	-2.20±0.74	-7.49±0.31	4.59 (2.7-7.7)	179.15(144.7-223.3)
20	9600	25.53±0.27	30.69±0.82	28.81±0.58	5.16±0.86	3.28±0.64	0.82±0.86	-3.78±0.64	0.57 (0.3-1.0)	13.69 (8.8-21.4)
21	10080	22.51±0.15	28.59±0.06	23.51±0.24	6.08±0.17	1.00±0.29	1.74±0.17	-6.06±0.29	0.30 (0.3-0.3)	66.72 (54.7-81.4)
22	10560	17.98±0.09	23.75±2.15	18.39±0.19	5.77±2.15	0.41±0.21	1.43±2.15	-6.65±0.21	0.37 (0.1-1.7)	100.43(86.8-116.2)

The $2^{-\Delta\Delta CT}$ values relative to the 2880 ADH values expression results were plotted against ADH for both arylphorin receptor and primer g (Figures 17.7 and 17.8).

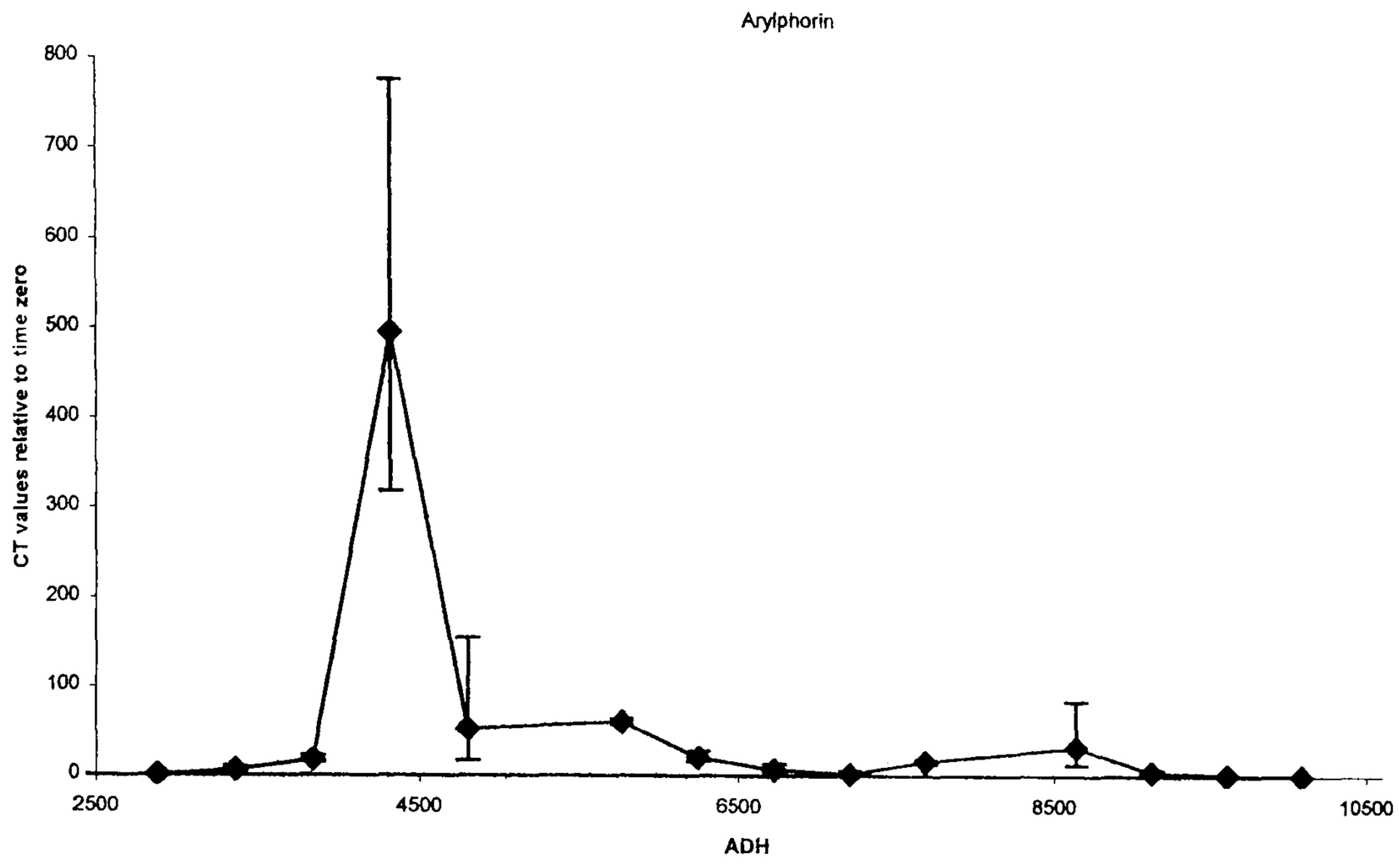

Figure 17.7. CT values relative to Time Zero (Day 6) as an indication of arylphorin receptor gene expression during the pupal phase. The error bars indicate the range as calculated from maximum and minimum ΔΔCT values.

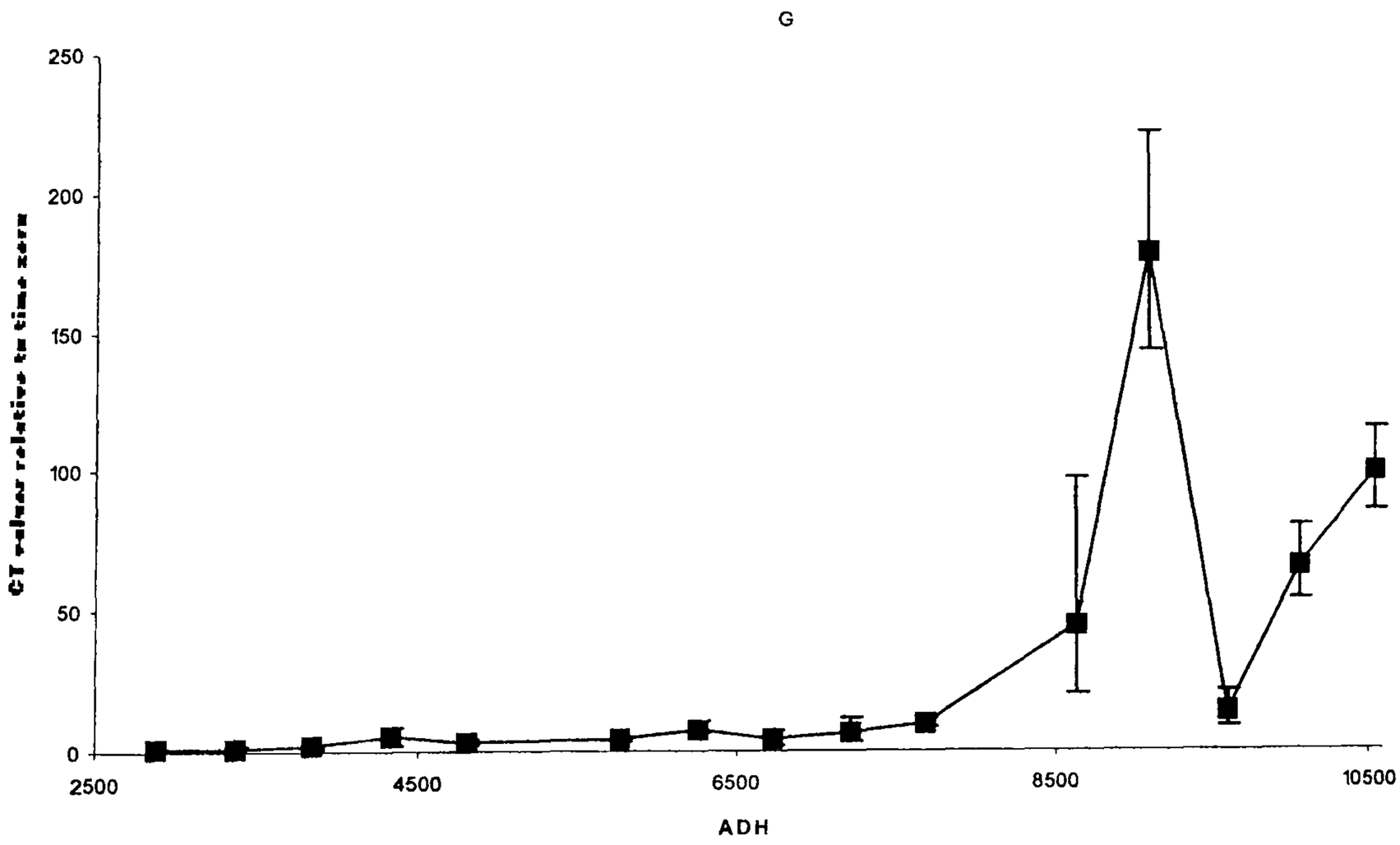

Figure 17.8. CT values relative to Time Zero (Day 6) as an indication of primer g gene expression during the pupal phase. The error bars indicate the range as calculated from maximum and minimum ΔΔCT values.

The graphs in Figures 17.7 and 17.8 illustrate that the maximum gene expression of the two genes is located at opposite ends of the *C. vicina* pupal phase. The CT values for arylphorin receptor after normalisation are that compared to the pre-pupal stage, there is a definite dramatic increase in expression on Day 9 (4320 ADH). The increase is 500 times that of the background level set as Day 6. This decreases sharply but still has 52-60 times greater expression at 4800 and 5760 ADH timepoints. This gradually decreases until 8640 ADH where it increases to around 33 times that of before the pupal stage. It decreases again until the last three timepoints (9600, 10080 and 10560 ADH) have lower arylphorin receptor gene expression than the 2880 ADH timepoint.

Primer g has levels of expression equivalent to that before the pupal phase until 7680 ADH, after which the level increases dramatically, reaching a peak at 9120 ADH of around 180 times that of the 2880 ADH value. The expression falls briefly then rises until adult emergence. At the end of the pupal phase primer g gene expression is 100 times that of the pre pupal stage figure.

17.2.2.2 LSP-2

As the LSP-2 primers/probe efficiencies were not equal to the actin primers and probe, the LSP-2 standard curves established in Section 17.2.1 would be used to establish an approximation for the amount of cDNA relative to the pooled cDNA sample. These values were normalised against similarly calculated actin values and then all pupal sample timepoints were compared with larval 2880 ADH (Table17.5).

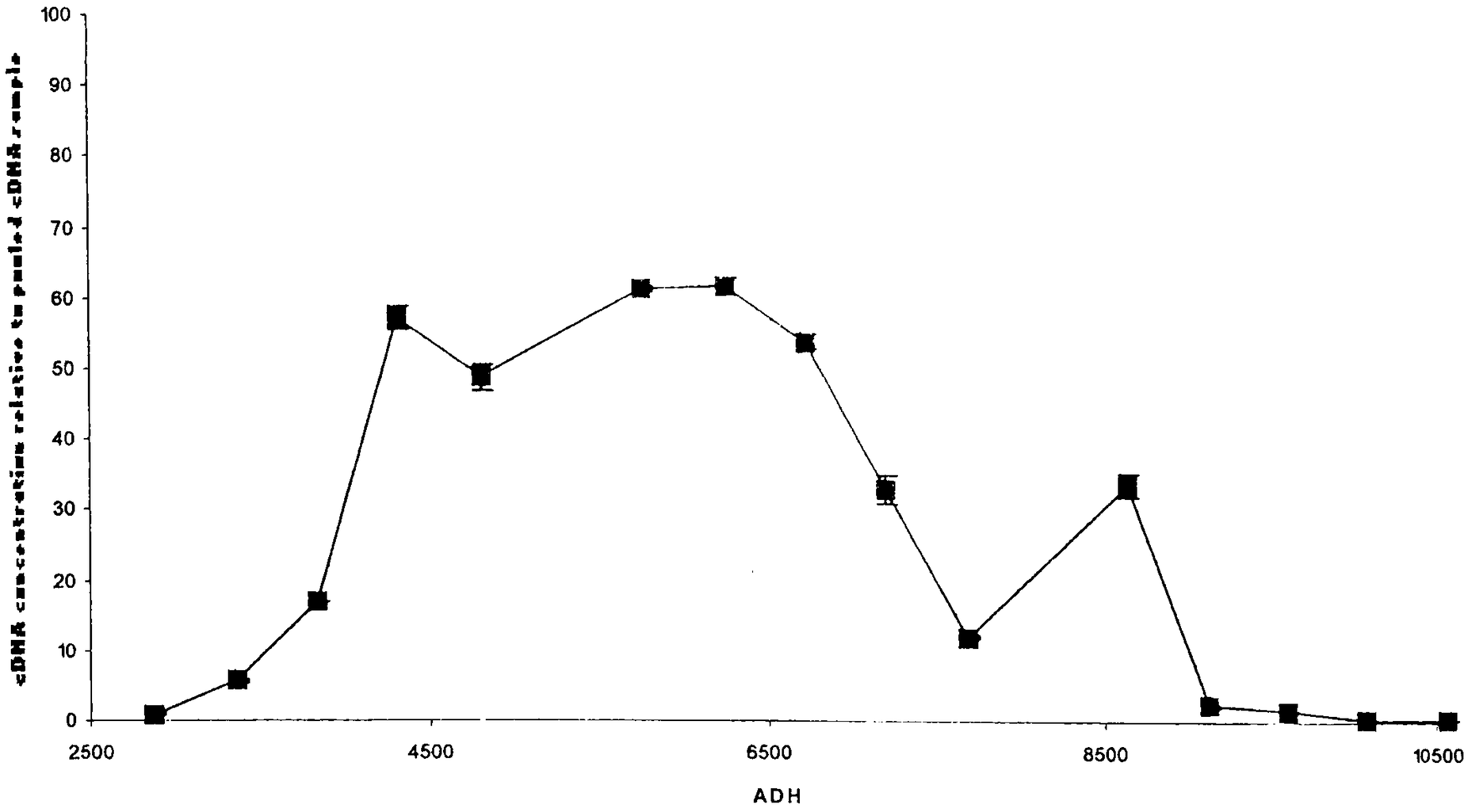

Figure 17.9. CT values relative to Time Zero (Day 6) as an indication of LSP-2 gene expression during the pupal phase. The error bars indicate the range as calculated from maximum and minimum ΔΔCT values.

The expression of LSP-2 increases at the beginning of the pupal phase, reaching a peak of 61 times the expression of the prepupal value at days 12 and 13 (5760 and 6240 ADH). After this, the gene expression gradually decreases to 12 times greater than the prepupal value at 7680 ADH. There is a brief increase to 33 times that of Day 6 at 8640 ADH before dramatically decreasing at the end of the pupal phase to less expression than that of prepupal stage.

Table 17.5. Normalised CT values for LSP-2 with Actin expression compared with Day 6 for *C. vicina* pupae.

Day	ADH	Average CT		Log[dilution cDNA]		Amount cDNA relative to pooled cDNA sample		Normalised LSP-2 value (LSP-2/Actin)	Normalised LSP-2 value relative to Day 6
		Actin	LSP-2	Actin	LSP-2	Actin	LSP-2		
6	2880	16.86±0.618	21.71±0.232	0.59	-0.37	3.88	0.43	0.11±0.004	1.00±0.038
7	3360	17.42±0.195	19.21±0.354	0.43	0.22	2.67	1.66	0.62±0.013	5.66±0.122
8	3840	19.24±0.038	19.43±0.091	-0.10	0.17	0.80	1.48	1.85±0.009	16.86±0.086
9	4320	21.75±0.541	20.27±0.334	-0.83	-0.03	0.15	0.94	6.26±0.187	56.91±1.698
10	4800	22.73±0.427	21.76±0.724	-1.11	-0.38	0.08	0.42	5.35±0.204	48.61±1.858
12	5760	23.08±0.024	21.77±0.149	-1.21	-0.38	0.06	0.41	6.71±0.046	61.03±0.423
13	6240	21.00±0.165	19.20±0.318	-0.61	0.22	0.25	1.67	6.77±0.124	61.59±1.129
14	6720	20.91±0.448	19.33±0.072	-0.58	0.19	0.26	1.56	5.93±0.129	53.91±1.173
15	7200	28.08±1.058	29.02±1.459	-2.65	-2.09	0.00	0.01	3.62±0.228	32.96±2.071
16	7680	19.35±0.232	20.18±0.064	-0.13	-0.01	0.74	0.98	1.33±0.016	12.07±0.150
18	8640	28.90±0.953	29.97±0.974	-2.89	-2.32	0.00	0.00	3.72±0.172	33.85±1.567
19	9120	19.66±0.162	23.63±0.154	-0.22	-0.82	0.60	0.15	0.25±0.003	2.28±0.024
20	9600	25.53±0.269	31.35±1.435	-1.92	-2.64	0.01	0.00	0.19±0.009	1.71±0.080
21	10080	22.51±0.153	31.08±0.874	-1.04	-2.58	0.09	0.00	0.03±0.001	0.27±0.008
22	10560	17.98±0.089	24.90±0.878	0.26	-1.12	1.84	0.08	0.04±0.001	0.37±0.013

17.2.3 Negative Controls

As the raw data in Appendix XI, Table 17.2 and Table 17.3 illustrate, some of the negative controls for the actin gene products had CT values. The fluorescence for these samples was such that it could be differentiated from the baseline and crossed the designated threshold limit. After the initial appearance of a positive negative control the plate was rerun but the water sample again showed some fluorescence with the actin primer/probe set.

17.3 Discussion

17.3.1 Negative Controls

The first consideration of this work was the presence of positive values for reactions that were either water or RNA samples. In theory, any results for samples run alongside positive negative controls should automatically be discarded and the experiment repeated. As mentioned in Section 17.2.3 the plate was reloaded and rerun and the negative reagent (ddH_2O) control was positive. It is possible therefore that the reagents had become contaminated. If the positive values had only been present in the RNA samples it could be concluded that the genomic DNA had not all been removed during the DNase step post RNA extraction, and this would have demanded returning to the RNA samples and repeating the DNA removal stage. Interestingly these positive negative controls are mainly present with the actin primer and probe set. This could be due to actin being present at consistently high levels in cells.

According to Bustin and Nolan (2004), there are no defined guidelines for interpreting a positive no template negative control. They suggest that if the CT value for the negative control is greater than 5 from the samples then is can be regarded as not caused by any DNA contaminant within the cDNA samples. The negative controls of the first actin dilution series (Table 17.2) have CT values greater that 5 from the 1 in 10,000 dilution. In the second (Table 17.3) the negative control CT value is 2.4 greater than the 1 in 10,000. Whilst this is within 5 units it should be noted that the neat cDNA sample is 16.18 less than the CT value for the negative control. If the trendline equation from the graph in Figure 17.1 is used with the no template control CT values for actin, it produces dilutions of 1 in 113,000 and 1 in 56,000.

Bustin and Nolan further advise that if the CT values of the unknowns are above 33-35, then the negative control must always be negative for the results to be valid. None of the CT values of the pupal timepoint samples in this study were that high.

There is reason therefore to continue with these results and as commented by Bustin and Nolan, presence of positive negative reagent controls should be noted in the summation of results but in some cases, as here, they can be disregarded as not interfering with the sample results.

17.3.2 Arylphorin receptor expression during the pupal phase

The results from this experiment indicate a definite increase in arylphorin receptor expression during the beginning of the pupal stage. At timepoint 4320 ADH the expression is nearly 500 times greater than before the pupal phase. The results of this work indicate that the level decreases to below that of the prepupal value at the end of the pupal stage (for the last three timepoints 9600, 10080 and 10560 ADH). This partly concurs with Burmester and Scheller (1995) who noted no mRNA in *C. vicina* adults . However Burmester and Scheller found that the arylphorin receptor was expressed from 2208 ADH (calculated from 4 days at 23 °C) until pupation. Whilst these data do not dispute this (the larval stages from 2208 ADH were not analysed) they have also shown that there is a peak in expression near the beginning of the pupal stage. Indeed whilst Burmester and Scheller declare that the protein production and therefore gene expression ceases at pupation, they do not appear to have included the pupal stage in their work (Burmester and Scheller 1995).

If the results of Burmester and Scheller are used in conjunction with those found here and arylphorin receptor expression begins at 2208 ADH then during the pupal stage the peak of expression will actually be greater than 500 times, as this work used the 2880 ADH value as 'Time Zero'.

17.3.3 Primer g gene expression during the pupal phase

This research has shown that the primer g gene is expressed at a greater level toward the end of the pupal stage. Levels remain similar to the prepupal stage until 8640

ADH when this particular mRNA becomes more prevalent. It has a gene expression level of around 180 times that of the prepupal level.

The gene known in this study as primer g showed homology to insects' muscle specific myosin heavy chain (MHC) sequences in GenBank (Chapter 15). This work has included no experiments to assess whether this is indeed one of the *C. vicina* muscle specific myosin genes. If this is the muscle specific myosin gene it could be postulated that its sudden increase in transcription toward the end of the pupal stage is due to the production and assembly of the muscles in the final adult structure. Berstein *et al.* (1986) examined the transcription of *D. melanogaster* thorax-specific MHC and found it began in the embryo and was present during the larval stages. During the early pupal stage, when larval structures were being broken down the MHC mRNA was not present. It accumulated during the mid-late pupal stage and continues being transcribed, albeit it lower levels, during in the adults. The work here on the expression of primer g gene concurs with their findings for expression of muscle specific myosin during the pupal stage.

It does not quite agree with the findings of Houlihan and Newton (1978 and 1979b) who were examining the changes to flight muscles in *C. vomitoria* during the pupal stage. Comparison of their results with the data here is hampered by the fact that they measured time as days since white puparium formation and not for oviposition. Also whilst the authors kept the pupae at 25 °C, the source of the *C. vomitoria* specimens was from a commercial supplier during the wandering larval three stage, thus it cannot be ascertained the exact degree hours accumulated by these insects. However, comparing time since white puparium formation with that of the insects in this study, Houlihan and Newton show there is a rapid increase in muscle filament size at approximately 7560 ADH (calculated by 25 °C x 7d x 24h + 3360ADH). This is before the mRNA increase noted in this study for primer g gene.

Whether primer g is the muscle specific myosin gene or not is irrelevant to this study as simply temporal molecular markers are being located, not new genes defined.

The analysis of the arylphorin receptor and the primer g gene have produced two markers – one whose increased expression defines early pupal phase (arylphorin receptor) and the other's increase gene expression defines the end before adult emergence (primer g).

17.3.4 LSP-2 expression during the pupal phase

After testing the primer/probe efficiencies it became obvious that the LSP-2 primers/probes were not as efficient as the other two primer/probe sets. The regression line of the serial dilution against CT value (Figure 17.4) for LSP-2 was –4.32, whereas the other genes were closer to the optimum value of –3.32. This indicates that for the LSP-2 set that DNA quantity is not doubling as would be expected in the exponential phase. This is further illustrated when the CT values for LSP-2 are compared to those of actin. If the primer/probe sets are working with similar efficiency the difference between the values should theoretically be zero. This was not the case for the LSP-2 gene. Whilst the results using this primer/probe set are still valid they cannot be used with the $2^{-\Delta\Delta CT}$ method of comparing CT values as this equation is based upon equal efficiencies. Repeating this work using a newly designed primer/probe set would be valuable to assess whether the trends noted in this work are still evident using the $2^{-\Delta\Delta CT}$ method.

The findings of this study regarding transcription of LSP-2 do not agree with that of Burmester *et al.* (1998) who noted that although the protein was present during the larval three and pupal stages, transcription of the gene was limited to the third larval stage. This work has indicated that not only is the gene being transcribed during the pupal stage it is at higher level than at the end of the third larval stage. Indeed at its transcription peak it reaches about 60 times that of the end of the third larval stage. The difference in findings may be due to the difference in methodologies as Burmester *et al.* used Northern blotting to examine transcription.

Interestingly Burmester *et al.* (1998) demonstrated the interaction of the arylphorin receptor with LSP-2. The findings of this study show a large increase in arylphorin receptor gene expression simultaneously as an increase and steady production of LSP-2. Production of the receptor is in synchrony with the production of its ligand.

As the levels of LSP-2 are not quite as significant as those of arylphorin receptor and primer g and more importantly as it remains at an approximately constant level from 4320 – 7200 ADH, the presence of LSP-2 increased expression when compared to the prepupal stage does not provide a viable molecular marker. At 20 °C, it cannot differentiate between days 9-15 – a large proportion of the pupal phase. The decrease

of gene expression at the end of the pupal stage just before adult emergence when compared is perhaps the only situation whereby it could on its own provide an entomologist with any indication of pupal age. It could however be useful to an entomologist if, after first analysing the arylphorin receptor and primer g expression levels and discovering that the unknown pupae is neither early nor late pupal stage to confirm that it is mid stage. Although, as mentioned previously, it could only estimate it as 4320-7200 ADH.

Chapter 18

Discussion and Conclusions of Part II

The purpose of Part II of this work was to assess the expression of potential molecular markers and how this expression can be related to pupal age. After RNA extraction and cDNA synthesis, gene regions were amplified and quantified either after conventional PCR or during real time PCR.

Different RNA extraction methods were examined in preliminary work on *Calliphora* pupal samples. TRIzol reagent along with Phase lock gel tubes followed by DNA-*free*™ DNase kit provided an efficient method to produce undegraded RNA with no residual genomic DNA.

Ideally each pupa would have been examined individually from RNA extraction to real time PCR quantification but this was not practical time or cost wise. Instead the RNA extracts were pooled within each timepoint with equal amounts of RNA to ensure that each individual pupal sample was equally represented.

There were three possibilities when selecting primers for cDNA synthesis - random hexamers, oligo dT or gene specific primers. Oligo dT primers were selected as these will replicate the mRNA in a sample because they anneal to the polyadenylated 3'tail on most mRNA. Gene specific primers are useful if the target mRNA is very rare but cDNA needs to be synthesised for every gene to be examined which in this study is impractical. The use of oligo dT or random hexamers allows cDNA to be synthesised once per experimental condition and specific genes amplified from this. The disadvantage of random hexamers is that when total RNA is used as a template, the majority of the sample consists of rRNA, and so most of cDNA will be synthesised by reverse transcribing this sequence. Due to there being a finite amount of cDNA that can be synthesised in a reverse transcription reaction, any mRNA fragments that are at a low level within the population may not be represented in the synthesised cDNA.

Actin was used as a normalisation gene for the analysis in this work. As mentioned in Chapter 13, several housekeeping genes could have be chosen as normalisation genes

for this work. 18S and 28S RNA genes were not chosen as they do not have 3' poly-A stretches and therefore cDNA synthesis using oligo dT primers would not be appropriate. As it would have been impractical and also have introduced unnecessary variation to use one set of primers for the housekeeping genes and oligo dT primers for the other gene region, 18S or 28S RNA genes were not utilised in this work.

There has been some controversy in the literature about the assumption that the housekeeping genes do not vary under experimental conditions. A recent paper (Dheda *et al.* 2004) found that in human blood samples, the common housekeeping genes β actin and GAPDH were found to vary between healthy and patients with tuberculosis. The authors note that this might only be the case for these particular samples and that further work needs to be carried out to assess for variability in other tissue types. However, according to Thellin *et al.* (1999), over 90% of published real time PCR experiments use the most common housekeeping genes, actin, tubulin, GAPDH and rRNA genes.

Raff *et al.* (1997) indicated that some β actin primers used for amplification of human cytoplasmic actin actually amplify pseudogenes located within the nuclear genome. The expression of these pseudogenes, which have no functional protein product, cannot be used to normalise gene expression. This could influence the results obtained in this study if the same were true for dipteran cytoplasmic actin. However, as these pseudogenes will naturally only be present in genomic DNA and not cDNA, they would only be amplified if the RNA samples were contaminated with genomic DNA. As shown in this experiment with negative controls, there was no residual DNA present after use of DNase.

The actin sequences produced in this study for *C. vicina* were the first Calliphoridae actin sequences deposited in GenBank.

Three potential molecular markers already characterised in the literature were used in this study, calliphorin, arylphorin receptor and LSP-2. Whilst these genes had been examined previously for temporal expression, none had been used as a method for determining insect age.

When deciding upon primers to amplify gene regions from cDNA samples, it is often advisable to choose primers that flank an intron. This will produce amplicons of differing lengths depending on whether DNA or cDNA is the template, thus providing an indication of any potential genomic DNA contamination. This was possible for calliphorin, which contains a small intron (59 bp) as demonstrated by Naumann and Scheller (1991). For the other gene region requiring primers to be designed (LSP-2), only the cDNA sequence was available in GenBank. The length variations between cDNA and genomic DNA would only have been evident in the endpoint quantification on agarose gels or by capillary electrophoresis, as the ABI 7700 (real time PCR machine) does not give any indication of amplicon length.

Differential display techniques were employed to design the other primers utilised in this work. This technique avoids the problems associated with random priming by the use of annealing control primers and also because once the DEGs have been elicited and sequenced then primers specifically for these gene regions can be designed. The use of differential display produced three DEGs (two were used in the quantification) using two different random primers. This technique provides a viable method of eliciting DEGs that can be potentially used as temporal markers.

Preliminary separation and quantification was conducted using capillary electrophoresis with further more accurate quantification carried out using real time PCR. When comparing the results found with endpoint and real time PCR generally they follow the same trends. It is possible with the endpoint results to assess when the gene regions are greater/less expressed. The real time results define the upregulation of gene regions, especially primer g and arylphorin receptor, to more distinct timepoints. *Calliphora vicina* arylphorin receptor gene shows definite upregulation at 4320 ADH. In this work this expression was about 500 times that of the expression in the late third larval stage. Likewise there is a distinct upregulation of primer g gene at the end of the pupal stage with expression levels at 9120 ADH about 180 times that of the third larval stage. For both of these markers, the range of expression at a particular timepoint was calculated relative to the third larval stage (Table 17.4). The range calculated was based upon the original standard deviation of CT values around the mean CT value. The lower value in the range provides the basis of a threshold level of expression, above which these genes can be nominated as 'highly expressed'.

Any discovered pupae of unknown age that indicate a large upregulation of these genes normalised to actin and relative to the larval three values established in this work could be assumed to be approximately these ages. Further work needs to be carried out to discover whether the actual values of upregulation relative to actin are always appropriate and a relative expression range for the maximum gene expression established.

It should be noted there appears to be a discrepancy with the endpoint quantifications and the real time quantifications for both these genes. If the timepoint for maximum expressions are compared, the maximum for primer g and arylphorin receptor after conventional PCR occurs at the timepoint later than when maximum expression is noted during real time PCR. Real time quantifications were only based upon one experimental set whereas the other quantifications were based upon both experimental sets. Whilst the quantifications taken after conventional PCR are less accurate, as discussed, it highlights the requirement to experiment further with more samples.

The LSP-2 region is upregulated throughout the middle part of the pupal stage (between 3840 and 8640 ADH). This region would be best used in conjunction with other markers, as it would be difficult to distinguish within this time period on the basis of LSP-2 alone. These markers could be molecular or not. As the observations of puparium colour indicate (Appendix IX), pupae younger than 3840 ADH (i.e. before the increase in LSP-2 gene expression) have not become fully darkened. Any darkened pupae with low LSP-2 expression will therefore be older than 8640 ADH. This marker can therefore distinguish pupae that have accumulated more than 8640 ADH from those younger.

The other markers used initially in this work but that were not carried through to real time quantification, calliphorin and primer h appeared to have similar expression profiles to LSP-2 and primer g respectively.

It could be postulated that the molecular markers discovered in this work may only be specific to the experimental conditions applied in this study. The insects were kept at a constant 20°C with ample food for the number of larvae in each cohort. In theory

the genes utilised in this study can be applied to pupae kept at different temperatures by calculating the appropriate value of ADH.

These molecular markers therefore provide the basis on which other markers for the pupal stage can be added to distinguish between more timepoints than were established in this work. This temporal gene expression research forms the beginning of potential further work on locating markers for the pupal stage.

18.1 Further work on gene expression

18.1.1 Developmental stages and species

This gene expression work should be extended to locate molecular markers to cover the whole developmental lifecycle of *C. vicina*. In this work pupae were sampled every 480 ADH. The duration between sampling times could be lowered to make the markers more specific.

Other Calliphoridae species could also be assessed. It is likely that the markers found for *C. vicina* would apply to closely related species. The LSPs (LSP-2, calliphorin and the receptor gene) were all originally characterised in *Drosophila* species, it is therefore probable that the types of genes expressed for development within Diptera have not fundamentally changed since divergence. The genes however should be assessed to see whether they were expressed at the same time and at the same level. It should be noted that primers designed for one species may not be viable for another.

18.1.2 Conditions

This work was carried out at a constant 20°C. Fluctuating temperature regimes could be experimented with to ensure the markers are present at the equivalent ADH of the constant temperatures.

Other experimental conditions that may be experienced upon the corpse, such as overcrowding, should also be subjected to gene expression analysis. As discussed in Chapter 1, competition for food produces undersized individuals and therefore it should be confirmed that factors such as this do not alter the timings of molecular marker expression.

18.1.3 Other potential markers

There are other genes that could now be examined to obtain further temporal markers for the *Calliphora* developmental lifecycle.

In insects the major steroid hormone that regulates development is called ecdysone (20-hydroxy ecdysone). The binding of ecdysone to its receptor initiates moulting, metamorphosis and reproduction (Hannan and Hill 1997). It is also thought to interact directly with RNA polymerase (Schenkel and Scheller 1982). Shaaya and Levenbrook (1982) note that in the third *C. vicina* larval stage, two peaks of ecdysone appear, a small one shortly before the mature larvae leave food and a main peak two days later which leads to pupariation. The first peak is mirrored by reabsorption of the storage proteins into the fat body (Myllek and Scheller 1984) however there is as yet no direct evidence for the influence of the hormones on the transcriptional activation of the arylphorin genes of *Calliphora* (Fischer and Scheller 1992). The binding of ecdysone to its receptor will begin the process of metamorphosis. In insects, ecdysone receptors appear to be made up of a dimer of two nuclear receptor proteins – ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP) (Hannan and Hill 2001 and references therein). Hannan and Hill (1997 and 2001) have sequenced and noted the interactions of the two genes EcR and USP for *L. cuprina*. The sequences can be found in GenBank accession numbers U75355 and AY007213 for EcR and USP respectively. Sequence analysis of the *L. cuprina* and *D. melanogaster* EcR indicated that they are homologues and that they demonstrated the same expression patterns during development – high levels are observed in embryonic, late larval and pupal stages (Hannan and Hill 1997). These two genes have therefore already been demonstrated as being differentially expressed throughout the lifecycle and are possible markers.

There is another family of genes that are influenced by the pulses of ecdysone during development. Ecdysone inducible genes (Eig) are a family of six genes that in *Drosophila* are expressed in the presence of ecdysone. White *et al.* (1999) used microarray technology to analyse *Drosophila* gene expression during metamorphosis and noted that the Eig are expressed after pupal formation but then get 'switched off' before adult emergence. The sequences for *D. melanogaster* have been deposited in GenBank. Again, these are potential markers in *Calliphora* species too.

Chitin is the major component of arthropod exoskeletons. Insect cuticles are secreted from the underlying epidermis. This synthesis of chitin is coordinated with the cycle of moults within the lifecycles of insects and is therefore under the control of the ecdysone hormone. According to Gagou *et al.* 2002, *D. melanogaster* chitin synthase genes A and B are expressed during periods of low ecdysone titres. This gene therefore has opposite expression patterns to the Eig genes mentioned previously. GenBank contains sequences for *D. melanogaster* and *L. cuprina* chitin synthase (Gagou *et al.* 2002; Tellam *et al.* 2000).

Garcia *et al.* (1996) examined the *white* gene in *L. cuprina*. This gene is a homologue of the *white* gene in *D. melanogaster* and is involved in eye pigment colouration. This gene has been demonstrated to be differentially expressed in both species with peaks in the third larval stage and pupal stages.

During development, dipteran muscle tissue will be synthesised to allow for the change in larval size and great amounts of muscle will be laid down in the developing fly. Lovato *et al.* (2001) and He and Haymer (1992) noted the temporal expression of muscle-specific actin genes in *Drosophila virilis* and *C. capitata* (respectively). Cytoplasmic actin, which was used as the housekeeping gene in the gene expression studies, should not be confused with the muscle-specific actins, which show variation in expression. Some are specific to the adult fly stage and are thus synthesised in the pupal and early adult stage. These actins are tissue specific too and as expected are especially located in the thorax (for wing movement) and legs. Other actins are expressed at differing levels during the whole lifespan. The muscle-specific actin family could also be examined as molecular markers for *Calliphora* development.

Whilst it is constructive to know which proteins are being synthesised during the *Calliphora* lifecycle, this does not necessarily indicate changes in gene expression. There are mechanisms that stop translation. Calliphorin mRNA was present within the pupal stage but as Schenkel and Scheller (1986) demonstrated through Western blot analysis, the protein was not synthesised during the pupal stage. For this reason, further work using differential display techniques could also complement the

examination of the gene regions suggested above. Also, assessment of the proteins themselves may provide other markers.

18.1.4 Examination of temporal protein synthesis

Houlihan (1976) demonstrated, using injections of cycloheximide during *C. vicina* pupal stage, that new proteins were still being made. This chemical inhibits protein synthesis by blocking the peptidyl synthetase activity of the ribosomes. This implies that for some structural changes *de novo* synthesis of protein is not required. In this case there will be new proteins present within the insect that analysing the mRNA will not discover. Analysis of proteins could therefore be complementary to the study of nucleic acids within the developing insect.

This could be achieved in several ways. If specific proteins were examined then antibodies could be raised and used in an ELISA. Along with the traditional protein separation techniques of SDS-PAGE and isoelectric focussing, other techniques such as GC/MS and LC/MS could be used to look for changes in the chemical composition of the insects with time. GC/MS has already been utilised on the black blowfly, *P.regina* to look for differences in cuticular hydrocarbons between populations (Byrne *et al.* 1995).

18.1.5 Microarray technology

As described in Chapter 11, the use of microarrays eliminates the requirement to examine specific differentially expressed genes. As discussed, no *Calliphora* array chips exist commercially, so to utilise this methodology a *C. vicina* gene library would need to be created and spotted onto chips for microarray analysis. Whilst this was beyond the realms of this study, it is still a viable experimental procedure for future analysis, especially as this work has provided evidence that differentially expressed genes can be used as temporal markers for forensic purposes.

18.1.6 mRNA longevity

An assumption made during this part of work was that the pupae were alive when they were sampled for RNA extraction. The most common method of examining pupal mortality is to note the numbers that have emerged as adults in a given amount of time (the length of which would depend upon the temperature). This was not possible for this experiment as pupae were sampled at specific timepoints and thus not allowed to

complete development. It was supposed that if any dead pupae were included in the experiment they would not contain any viable mRNA. mRNA is degraded within the cellular environment by ribonucleases. This phenomenon is an inbuilt method to control protein synthesis. The longer an mRNA molecule exists within the cytoplasm the more proteins will be translated using it as a template. The mRNA molecules from different genes have their longevity encoded into their sequence and this affects different mechanisms including the action of the intracellular enzymes. Some mRNA molecules have very short half-lives (5-10min).

After death the ribonucleases continue to digest the mRNA along with exogenous ribonucleases that are introduced from bacteria and the environment. It could therefore be assumed that any pupa that dies during the pupal stage will have little/no mRNA and would therefore not interfere with this experimentation if included. However, it should be noted that a few studies have shown that viable mRNA can be extracted from post-mortem tissues and that PCR on post-mortem cDNA could amplify fragment up to 904bp (Phang *et al.* 1994). Indeed one paper shows that mRNA degradation could possibly be used to estimate PMI for 4-5 days post-mortem (Bauer *et al.* 2003). However this work has been in done in mammals only (humans and rats) and studies tended to focus on brain tissue. Inoue *et al.* (2002) indicated that other human tissues showed a much faster mRNA degradation than brain tissue. Most tissues only have amplifiable mRNA present 24 hours after death. It is likely therefore that pupal mRNA will degrade quickly as expected.

It would be interesting however to examine the hourly mRNA postmortem degradation of blowfly and other forensically interesting Arthropods, similar to the experimental work of Inoue *et al.* (2002) in the dead rat body. One of the problems with discovery of dead insects on a corpse is that whilst a minimum PMI can be established it will not be as accurate as one based on live evidence. As stressed in Part I of this work, DNA identification can still occur after insect death, even in decomposing insects therefore it would be useful if an estimate of when an insect died could also be established from the evidence presented. It is likely however, as discussed above, that this technique would probably only be able to see whether the insect had recently died, but this will still increase the accuracy of the PMI established.

Chapter 19

Concluding Discussion

This work was divided into two parts; each addressed one of the challenges encountered by forensic entomologists when making estimations of PMI. The first part examined the identification of species and ultimately populations and the second assessed a method of more accurate age determination of immature stages.
Both located molecular markers for use with *C. vicina* and *C. vomitoria* blowfly species. This work has formed the basis for further studies to locate more markers both for population and age determination.

The ultimate endpoint of further work would be to produce a microarray chip containing not only oligonucleotides known to differentiate between species and populations but also those that will bind markers for various immature ages. Thus species, population and age could be determined simultaneously.

The alternative to use of microarray technology would be to produce an ELISA. Naturally these are based upon protein markers as opposed to DNA sequences and appropriate antibodies would need to be raised. Once successful the eventual ELISA 'product' would be an immunosorbent stick, which elicited a chromogenic change on binding of the specific antigen to antibody. This stick would be versatile enough to be used by a forensic investigator not experienced in molecular biology. Forensic science as a whole is moving towards producing more equipment to be used at the scene of crime and this is something that could potentially end up taken to a scene of crime.

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Appendix I.

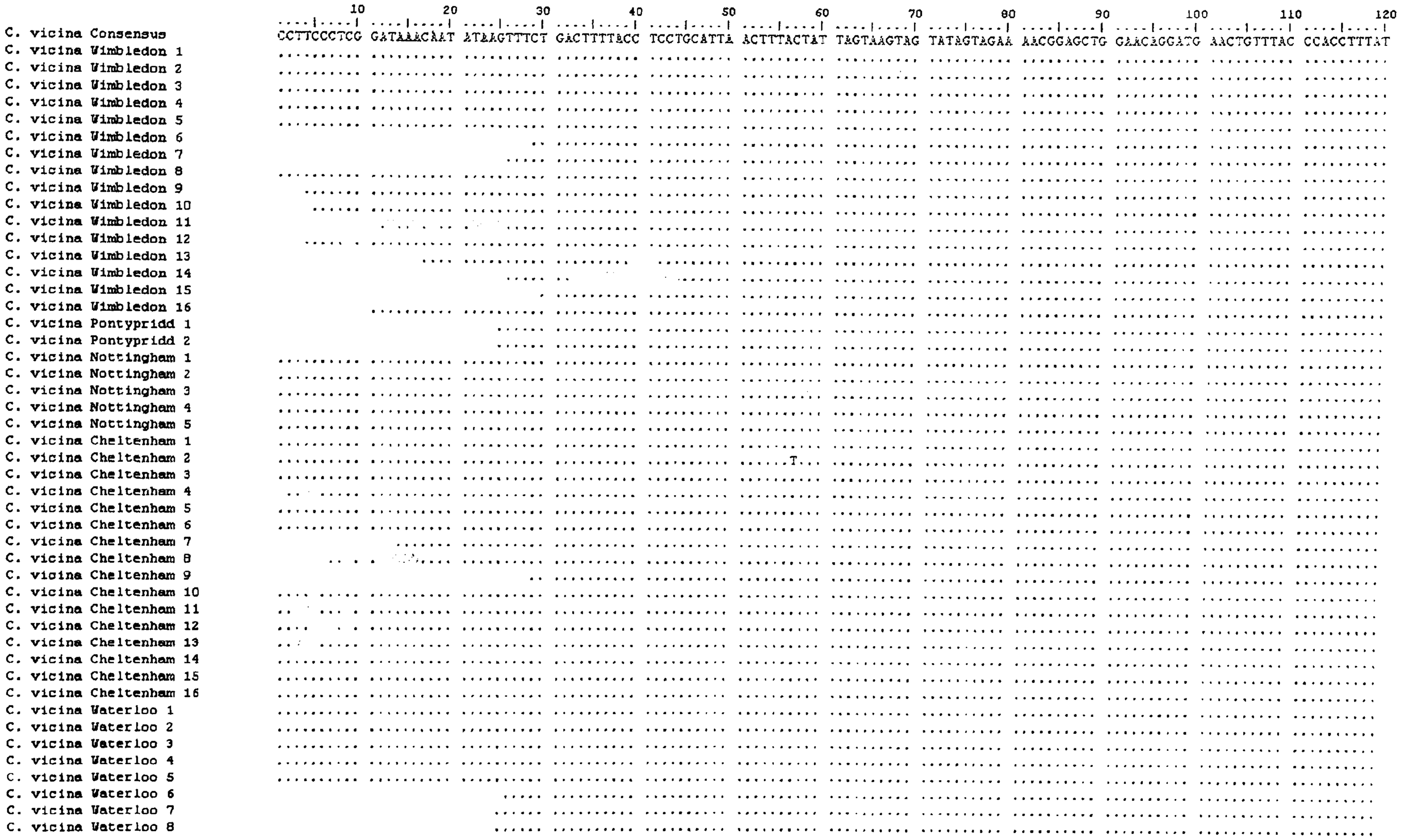
Time (hours) to reach developmental stages for *C. vicina* Cheltenham population for three replicate cohorts.

Larval 1 (h)			Larval 2 (h)			Larval 3 (h)			Pupal (h)			Adult (h)		
1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
19.25	18.75	18.25	32	32	32	80	80	80	189.5	187.5	179	524.5	515	515
20.25	19.25	19.25	32	32	32	80	80	80	189.5	188.5	187.5	524.5	523.5	515
20.75	19.75	19.25	32	39.5	32	80	80	80	190.5	188.5	189.5	525.5	523.5	523.5
20.75	19.75	19.25	39.5	40.5	39.5	80	80	87.5	191.5	189.5	189.5	526.5	525.5	523.5
21.25	20.75	19.75	39.5	41.5	39.5	80	80	87.5	191.5	190.5	189.5	526.5	525.5	523.5
21.75	20.75	20.25	39.5	41.5	39.5	80	87.5	87.5	193.5	190.5	190.5	527.5	525.5	525.5
21.75	21.25	20.25	40.5	42.5	40.5	80	87.5	87.5	193.5	190.5	190.5	529.5	526.5	526.5
21.75	21.25	20.25	40.5	42.5	40.5	80	87.5	87.5	193.5	191.5	192.5	531.5	527.5	527.5
21.75	21.75	20.75	40.5	42.5	40.5	87.5	88.5	88.5	193.5	193.5	193.5	539	527.5	527.5
22.25	21.75	20.75	40.5	42.5	40.5	87.5	88.5	88.5	194.5	193.5	193.5	539	528.5	528.5
22.75	21.75	21.25	41.5	42.5	40.5	87.5	88.5	88.5	194.5	194.5	194.5	539	528.5	528.5
22.75	22.25	21.25	41.5	42.5	40.5	87.5	88.5	88.5	195.5	195.5	194.5	539	528.5	529.5
23.25	22.25	21.25	41.5	42.5	41.5	87.5	88.5	88.5	195.5	195.5	194.5	539	530.5	529.5
23.25	22.25	21.75	41.5	42.5	41.5	87.5	88.5	89.5	203	195.5	195.5	539	531.5	529.5
23.25	22.25	21.75	41.5	42.5	41.5	87.5	88.5	89.5	203	203	195.5	546.5	531.5	529.5
23.75	22.25	22.25	41.5	43.5	42.5	88.5	89.5	89.5	210.5	203	203	546.5	539	530.5
23.75	22.75	22.25	42.5	43.5	42.5	88.5	89.5	89.5	210.5	203	203	546.5	539	530.5
23.75	22.75	22.25	42.5	43.5	42.5	88.5	89.5	89.5	210.5	203	203	547.5	539	530.5
23.75	22.75	22.25	43.5	43.5	42.5	88.5	90.5	89.5	211.5	203	203	547.5	539	531.5
24.25	22.75	22.75	43.5	43.5	42.5	89.5	90.5	89.5	211.5	210.5	203	548.5	539	531.5
24.25	22.75	22.75	43.5	43.5	42.5	89.5	90.5	89.5	211.5	210.5	210.5	548.5	546.5	531.5
24.75	23.25	22.75	43.5	43.5	42.5	89.5	90.5	89.5	212.5	210.5	210.5	549.5	546.5	531.5
24.75	23.25	22.75	43.5	44.5	42.5	89.5	90.5	90.5	212.5	210.5	210.5	549.5	546.5	539
24.75	23.25	22.75	44.5	44.5	43.5	89.5	91.5	90.5	213.5	210.5	210.5	549.5	547.5	539
25.25	23.25	22.75	44.5	44.5	43.5	89.5	91.5	90.5	213.5	211.5	210.5	550.5	547.5	539
25.25	23.75	23.25	44.5	44.5	43.5	90.5	91.5	90.5	215.5	211.5	211.5	551.5	547.5	539
25.75	23.75	23.25	45.5	44.5	43.5	90.5	92.5	90.5	216.5	211.5	211.5	551.5	548.5	539
25.75	23.75	23.25	45.5	45.5	43.5	90.5	93.5	90.5	216.5	212.5	212.5	551.5	548.5	539
25.75	23.75	23.25	45.5	45.5	43.5	90.5	93.5	90.5	216.5	213.5	212.5	552.5	548.5	539
26.25	24.25	23.75	47.5	46.5	44.5	90.5	93.5	90.5	218.5	214.5	212.5	552.5	548.5	539
26.25	24.25	23.75	47.5	46.5	44.5	91.5	93.5	91.5	218.5	214.5	212.5	552.5	548.5	546.5
26.25	24.25	23.75	47.5	46.5	44.5	92.5	94.5	91.5	219.5	214.5	212.5	553.5	549.5	546.5
26.75	24.75	24.25	48.5	47.5	44.5	92.5	94.5	91.5	227	216.5	212.5	563	549.5	546.5
26.75	24.75	24.25	48.5	48.5	44.5	92.5	95.5	91.5	227	217.5	213.5	563	549.5	547.5
26.75	24.75	24.25	48.5	48.5	44.5	94.5	104	92.5	227	217.5	213.5		563	547.5
26.75	24.75	24.25	48.5	56	44.5	95.5	104	92.5	227	218.5	213.5			548.5

27.75	24.75	24.25	56		44.5	104		92.5		218.5	214.5			550.5
	25.25	24.75			45.5			93.5		219.5	214.5			551.5
	25.25	24.75			45.5			93.5		227	214.5			551.5
	25.75	24.75			46.5			94.5		227	215.5			554.5
	25.75	25.25			46.5			94.5		227	215.5			563
	26.25	25.25			47.5			96.5		227	216.5			563
	27.25	25.25			47.5			104		227	217.5			
	27.25	25.25			56						217.5			
	27.25	25.75			56						217.5			
		25.75									219.5			
		26.75									219.5			
		26.75									219.5			
		26.75									227			

Appendix II.

Alignment of *C. vicina* and *C. vomitoria* Cytochrome oxidase I samples. Dot indicates identical nucleotide to *C. vicina* consensus sequence. Ruler denotes base numbering relative to this sequence and not position within mitochondrial genome.



C. vicina Waterloo 9
C. vicina Waterloo 10
C. vicina Waterloo 11
C. vicina Waterloo 12
C. vicina Waterloo 13
C. vicina Waterloo 14
C. vicina Waterloo 15
C. vicina Waterloo 16
C. vicina Waterloo 17
C. vicina Waterloo 18
C. vicina Waterloo 19
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C. vicina Waterloo 24
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C. vicina Waterloo 26
C. vicina Waterloo 27
C. vicina Waterloo 28
C. vicina Waterloo 29
C. vicina Waterloo 30
C. vicina Waterloo 31
C. vicina Waterloo 32
C. vicina Waterloo 33
C. vicina Nonsuch Park 1
C. vicina Nonsuch Park 2
C. vicina Nonsuch Park 3
C. vicina Nonsuch Park 4
C. vicina Greenwich 1
C. vicina Greenwich 2
C. vicina Greenwich 3
C. vicina Greenwich 4
C. vicina Greenwich 5
C. vicina Greenwich 6
C. vicina Snaresbrook 1
C. vicina Snaresbrook 2
C. vicina Snaresbrook 3
C. vicina Stanmore 1
C. vicina Stanmore 2
C. vicina Stanmore 3
C. vicina Stanmore 4
C. vicina Stanmore 5
C. vicina Stanmore 6
C. vicina York 1
C. vicina York 2
C. vicina York 3
C. vicina York 4
C. vicina York 5
C. vicina Keswick 1

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C. vicina Keswick 2
C. vicina Keswick 3
C. vicina Keswick 4
C. vicina Keswick 5
C. vicina Great Yarmouth 1
C. vicina Great Yarmouth 2
C. vicina Great Yarmouth 3
C. vicina Great Yarmouth 4
C. vicina Great Yarmouth 5
C. vicina Derby 1
C. vicina Mitcham Junction 1
C. vicina Mitcham Junction 2
C. vicina Mitcham Junction 3
C. vicina Mitcham Junction 4
C. vicina Larva 1
C. vicina Larva 2
C. vicina Larva 3
C. vicina Larva 4
C. vicina Larva 5
C. vicina Larva 6
C. vicina Larva 7
C. vicina Larva 8
C. vomitoria Consensus          .A.....A.....T..........T.....T
C. vomitoria WimbledonCommon1   .A.....A.....T..........T.....T
C. vomitoria WimbledonCommon2        A.....T..........T.....T
C. vomitoria WimbledonCommon3        A.....T..........T.....T
C. vomitoria Hamstead Heath 1        A.....T..........T.....T
C. vomitoria Hamstead Heath 2        A.....T..........T.....T
C. vomitoria Hamstead Heath 3        A.....T..........T.....T
C. vomitoria Hamstead Heath 4        A.....T..........T.....T
C. vomitoria York 1             .A.....A.....T..........T.....T
C. vomitoria Nonsuch Park 1                             T.....T
C. vomitoria Nonsuch Park 2                             T.....T
C. vomitoria Nonsuch Park 3                             T.....T
C. vomitoria Nonsuch Park 4                             T.....T
C. vomitoria Nonsuch Park 5                             T.....T
C. vomitoria Nonsuch Park 6                             T.....T
C. vomitoria Nonsuch Park 7                             T.....T
C. vomitoria Nonsuch Park 8                             T.....T
C. vomitoria Keswick 1          .A.....A.....T..........T.....T
C. vomitoria Keswick 2          .A.....A.....T..........T.....T
C. vomitoria Keswick 3          .A.....A.....T..........T.....T
C. vomitoria Keswick 4          .A.....A.....T..........T.....T
C. vomitoria Keswick 5          .A.....A.....T..........T.....T
C. vomitoria Petts Wood 1                               T.....T
C. vomitoria Petts Wood 2                               T.....T
C. vomitoria Petts Wood 3                               T.....T
C. vomitoria Boxhill 1                                  T.....T
C. vomitoria Boxhill 2               A.....T..........T.....T
C. vomitoria East Sheen 1                               T.....T
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C. vomitoria East Sheen 2                  ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria East Sheen 3                    .. ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria East Sheen 4                     . ..... ..... ......T... ..... ..... ..... ....T..... .........T .....
C. vomitoria East Sheen 5                   ... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Mitcham Junction1  .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Mitcham Junction2  .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Mitcham Junction3  .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Mitcham Junction4  .A..NNN... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Mitcham Junction5                                    ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Stanmore 1         .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Stanmore 2                     .. ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Stanmore 3                      ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Stanmore 4                     ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Cheltenham 1                     ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria Cheltenham 2                     ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 1                  .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 2                  .A..... A.....T... ..... ..... ..... ......T... ..... ..... ..... ....T..... .........T .....
C. vomitoria 3                  .A..... A.....T... ..... ..... ..... ......T... ..... ..... ..... ....T..... .........T .....
C. vomitoria 4                  .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 5                  .A..... A.....T... ..... ..... ..... ......T... ..... ..... ..... ....T..... .........T .....
C. vomitoria 6                  .A..... A.....T... ..... ..... ..... ......T... ..... ..... ..... ....T..... .........T .....
C. vomitoria 7                  .A..... A.....T... ..... ..... ..... ......T... ..... ..... ..... ....T..... .........T .....
C. vomitoria 8                  .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 9                  .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 10                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 11                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 12                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 13                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 14                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 15                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 16                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 17                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 18                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 19                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 20                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 21                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 22                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 23                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 24                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 25                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 26                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 27                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 28                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 29                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 30                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 31                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 32                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 33                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 34                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
C. vomitoria 35                 .A..... A.....T... ..... ..... ..... ..... ..... ..... ..... ....T..... .........T .....
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C. vomitoria 36          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 37          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 38          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 39          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 40          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 41          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 42          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 43          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 44          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 45          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 46          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 47          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 48          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 49          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 50          .A..... A.....T... .......... .......... .......... ......T... .......... .......... .......... ....T..... .........T ..........
C. vomitoria 51          .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 1      .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 2      .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 3      .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 4      .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 5      .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 6      .A..... A.....T... .......... .......... .......... ......T... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 7      .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 8      .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 9      .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 10     .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
C. vomitoria Larva 11     .A..... A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........

                                  130       140       150       160       170       180       190       200       210       220       230       240
                         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C. vicina Consensus      CTTCTAATAT CGCTCATGGA GGAGCTTCTG TTGATTTAGC TATTTTTTCT TTACACTTAG CAGGAATTTC TTCAATTTTA GGAGCTGTAA ATTTTATTAC TACAGTTATT AATATACGAT
C. vicina Wimbledon 1    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 2    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 3    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 4    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 5    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 6    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 7    .......... .......... .......... .......... .......... .......... .......... ..G....... .......... .......... .......... ..........
C. vicina Wimbledon 8    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 9    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 10   .......... .......... .......... .......... .......... .......... .......... ..G....... .......... .......... .......... ..........
C. vicina Wimbledon 11   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 12   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 13   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 14   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 15   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 16   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Pontypridd 1   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Pontypridd 2   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Nottingham 1   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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C. vicina Nottingham 2 ....
C. vicina Nottingham 3 ....
C. vicina Nottingham 4 ....
C. vicina Nottingham 5 ....
C. vicina Cheltenham 1 ....
C. vicina Cheltenham 2 ....
C. vicina Cheltenham 3 ....
C. vicina Cheltenham 4 ....
C. vicina Cheltenham 5 ....
C. vicina Cheltenham 6 ....
C. vicina Cheltenham 7 ....
C. vicina Cheltenham 8 ....
C. vicina Cheltenham 9 ....
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C. vicina Cheltenham 16 ....
C. vicina Waterloo 1 ....
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C. vicina Waterloo 5 ....
C. vicina Waterloo 6 ....
C. vicina Waterloo 7 ....
C. vicina Waterloo 8 ....
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C. vicina Waterloo 29 ....
C. vicina Waterloo 30 ....

C. vicina Waterloo 31 ....
C. vicina Waterloo 32 ....
C. vicina Waterloo 33 ....
C. vicina Nonsuch Park 1 ....
C. vicina Nonsuch Park 2 ....
C. vicina Nonsuch Park 3 ....
C. vicina Nonsuch Park 4 ....
C. vicina Greenwich 1 ....
C. vicina Greenwich 2 ....
C. vicina Greenwich 3 ....
C. vicina Greenwich 4 ....
C. vicina Greenwich 5 ....
C. vicina Greenwich 6 ....
C. vicina Snaresbrook 1 ....
C. vicina Snaresbrook 2 ....
C. vicina Snaresbrook 3 ....
C. vicina Stanmore 1 ....
C. vicina Stanmore 2 ....
C. vicina Stanmore 3 ....
C. vicina Stanmore 4 ....
C. vicina Stanmore 5 ....
C. vicina Stanmore 6 ....
C. vicina York 1 ....
C. vicina York 2 ....
C. vicina York 3 ....
C. vicina York 4 ....
C. vicina York 5 ....
C. vicina Keswick 1 ....
C. vicina Keswick 2 ....
C. vicina Keswick 3 ....
C. vicina Keswick 4 ....
C. vicina Keswick 5 ....
C. vicina Great Yarmouth 1 ....
C. vicina Great Yarmouth 2 ....
C. vicina Great Yarmouth 3 ....
C. vicina Great Yarmouth 4 ....
C. vicina Great Yarmouth 5 ....
C. vicina Derby 1 ....
C. vicina Mitcham Junction 1 ....
C. vicina Mitcham Junction 2 ....
C. vicina Mitcham Junction 3 ....
C. vicina Mitcham Junction 4 ....
C. vicina Larva 1 ....
C. vicina Larva 2 ....
C. vicina Larva 3 ....
C. vicina Larva 4 ....
C. vicina Larva 5 ....
C. vicina Larva 6 ....
C. vicina Larva 7 ....
C. vicina Larva 8 ....

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C. vomitoria Consensus          .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria WimbledonCommon1   .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria WimbledonCommon2   .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria WimbledonCommon3   .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Hamstead Heath 1                                                 .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Hamstead Heath 2   .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Hamstead Heath 3   .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Hamstead Heath 4   .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria York 1             .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Nonsuch Park 1     .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Nonsuch Park 2     .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Nonsuch Park 3     .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Nonsuch Park 4     .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Nonsuch Park 5     .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Nonsuch Park 6     .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Nonsuch Park 7     .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Nonsuch Park 8     .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Keswick 1          .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Keswick 2          .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Keswick 3          .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Keswick 4          .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Keswick 5          .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Petts Wood 1       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Petts Wood 2       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Petts Wood 3       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..C.......
C. vomitoria Boxhill 1          .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Boxhill 2          .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria East Sheen 1       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria East Sheen 2       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria East Sheen 3       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria East Sheen 4       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria East Sheen 5       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Mitcham Junction1  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Mitcham Junction2  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Mitcham Junction3  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Mitcham Junction4  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Mitcham Junction5  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Stanmore 1         .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Stanmore 2         .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Stanmore 3         .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Stanmore 4         .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Cheltenham 1       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Cheltenham 2       .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria 1                  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria 2                  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria 3                  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria 4                  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria 5                  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria 6                  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria 7                  .......... T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
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C. vomitoria 8          ..........T..A.....T.....
C. vomitoria 9          ..........T..A.....T.....
C. vomitoria 10         ..........T..A.....T.....
C. vomitoria 11         ..........T..A.....T.....
C. vomitoria 12         ..........T..A.....T.....
C. vomitoria 13         ..........T..A.....T.....
C. vomitoria 14         ..........T..A.....T.....
C. vomitoria 15         ..........T..A.....T.....
C. vomitoria 16         ..........T..A.....T.....
C. vomitoria 17         ..........T..A.....T.....
C. vomitoria 18         ..........T..A.....T.....
C. vomitoria 19         ..........T..A.....T.....
C. vomitoria 20         ..........T..A.....T.....
C. vomitoria 21         ..........T..A.....T.....
C. vomitoria 22         ..........T..A.....T.....
C. vomitoria 23         ..........T..A.....T.....
C. vomitoria 24         ..........T..A.....T.....
C. vomitoria 25         ..........T..A.....T.....
C. vomitoria 26         ..........T..A.....T.....
C. vomitoria 27         ..........T..A.....T.....
C. vomitoria 28         ..........T..A.....T.....
C. vomitoria 29         ..........T..A.....T.....
C. vomitoria 30         ..........T..A.....T.....
C. vomitoria 31         ..........T..A.....T.....
C. vomitoria 32         ..........T..A.....T.....
C. vomitoria 33         ..........T..A.....T.....
C. vomitoria 34         ..........T..A.....T.....
C. vomitoria 35         ..........T..A.....T.....
C. vomitoria 36         ..........T..A.....T.....
C. vomitoria 37         ..........T..A.....T.....
C. vomitoria 38         ..........T..A.....T.....
C. vomitoria 39         ..........T..A.....T.....
C. vomitoria 40         ..........T..A.....T.....
C. vomitoria 41         ..........T..A.....T.....
C. vomitoria 42         ..........T..A.....T.....
C. vomitoria 43         ..........T..A.....T.....
C. vomitoria 44         ..........T..A.....T.....
C. vomitoria 45         ..........T..A.....T.....
C. vomitoria 46         ..........T..A.....T.....
C. vomitoria 47         ..........T..A.....T.....
C. vomitoria 48         ..........T..A.....T.....
C. vomitoria 49         ..........T..A.....T.....
C. vomitoria 50         ..........T..A.....T.....
C. vomitoria 51         ..........T..A.....T.....
C. vomitoria Larva 1    ..........T..A.....T.....
C. vomitoria Larva 2    ..........T..A.....T.....
C. vomitoria Larva 3    ..........T..A.....T.....
C. vomitoria Larva 4    ..........T..A.....T.....
C. vomitoria Larva 5    ..........T..A.....T.....
C. vomitoria Larva 6    ..........T..A.....T.....
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                                250       260       270       280       290       300       310       320       330       340       350       360
                                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C. vomitoria Larva 7            ..........T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Larva 8            ..........T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Larva 9            ..........T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Larva 10           ..........T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........
C. vomitoria Larva 11           ..........T..A...... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........

C. vicina Consensus             CAACAGGAAT TACATTCGAC CGAATACCAT TATTTGTTTG ATCTGTAGTA ATTACAGCTT TATTACTTTT ATTATCTTTA CCAGTATTAG CAGGTGCTAT TACTATATTA TTAACAGATC
C. vicina Wimbledon 1           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 2           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 3           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 4           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 5           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 6           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 7           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 8           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 9           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 10          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 11          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 12          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 13          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 14          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 15          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 16          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Pontypridd 1          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Pontypridd 2          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Nottingham 1          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Nottingham 2          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Nottingham 3          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Nottingham 4          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Nottingham 5          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 1          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 2          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 3          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 4          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 5          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 6          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 7          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 8          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 9          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 10         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 11         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 12         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 13         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 14         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 15         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Cheltenham 16         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Waterloo 1            .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Waterloo 2            .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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C. vicina Waterloo 3 ....
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C. vicina Waterloo 29 ....
C. vicina Waterloo 30 ....
C. vicina Waterloo 31 ....
C. vicina Waterloo 32 ....
C. vicina Waterloo 33 ....
C. vicina Nonsuch Park 1 ....
C. vicina Nonsuch Park 2 ....
C. vicina Nonsuch Park 3 ....
C. vicina Nonsuch Park 4 ....
C. vicina Greenwich 1 ....
C. vicina Greenwich 2 ....
C. vicina Greenwich 3 ....
C. vicina Greenwich 4 ....
C. vicina Greenwich 5 ....
C. vicina Greenwich 6 ....
C. vicina Snaresbrook 1 ....
C. vicina Snaresbrook 2 ....
C. vicina Snaresbrook 3 ....
C. vicina Stanmore 1 ....
C. vicina Stanmore 2 ....
C. vicina Stanmore 3 ....
C. vicina Stanmore 4 ....
C. vicina Stanmore 5 ....
C. vicina Stanmore 6 ....

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C. vicina York 1                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 2                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 3                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 4                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 5                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 1             ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 2             ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 3             ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 4             ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 5             ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Great Yarmouth 1      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Great Yarmouth 2      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Great Yarmouth 3      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Great Yarmouth 4      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Great Yarmouth 5      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Derby 1               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Mitcham Junction 1    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Mitcham Junction 2    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Mitcham Junction 3    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Mitcham Junction 4    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Larva 1               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Larva 2               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Larva 3               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Larva 4               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Larva 5               ..........  ..........  ..........  ..........  G.........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Larva 6               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Larva 7               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Larva 8               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vomitoria Consensus          .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria WimbledonCommon1   .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria WimbledonCommon2   .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  .....
C. vomitoria WimbledonCommon3   .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Hamstead Heath 1   .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Hamstead Heath 2   .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Hamstead Heath 3   .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Hamstead Heath 4   .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria York 1             .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Nonsuch Park 1     .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Nonsuch Park 2     .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Nonsuch Park 3     .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Nonsuch Park 4     .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Nonsuch Park 5     .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Nonsuch Park 6     .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Nonsuch Park 7     .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ....  .....  ..........
C. vomitoria Nonsuch Park 8     .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Keswick 1          .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Keswick 2          .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Keswick 3          .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Keswick 4          .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
C. vomitoria Keswick 5          .......T..  ...C......  ..........  ..........  ...A......  ........C.  ..........  ..........  ..........  ....A.....  ..........  ..........
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C. vomitoria Petts Wood 1       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Petts Wood 2       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Petts Wood 3       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Boxhill 1          .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Boxhill 2          .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria East Sheen 1       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria East Sheen 2       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria East Sheen 3       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria East Sheen 4       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria East Sheen 5       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Mitcham Junction1  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Mitcham Junction2  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Mitcham Junction3  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Mitcham Junction4  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Mitcham Junction5  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Stanmore 1         .......T.. ...C....N. ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Stanmore 2         .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Stanmore 3         .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Stanmore 4         .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Cheltenham 1       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria Cheltenham 2       .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 1                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 2                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 3                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 4                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 5                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 6                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 7                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 8                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 9                  .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 10                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 11                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 12                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 13                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 14                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 15                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 16                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 17                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 18                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 19                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 20                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 21                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 22                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 23                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 24                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 25                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 26                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 27                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 28                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
C. vomitoria 29                 .......T.. ...C..... ......... ......... ...A..... ........C. ......... ......... ......... ....A..... ......... .........
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C. vomitoria 30          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 31          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 32          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 33          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 34          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 35          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 36          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 37          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 38          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 39          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 40          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 41          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 42          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 43          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 44          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 45          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 46          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 47          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 48          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 49          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 50          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria 51          .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 1      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 2      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 3      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 4      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 5      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 6      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 7      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 8      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 9      .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 10     .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........
C. vomitoria Larva 11     .......T.. ...C..... .......... .......... ...A..... ........C. .......... .......... .......... ....A..... .......... ..........

                                    370        380        390        400        410
                         ....|....| ....|....| ....|....| ....|....| ....|....| ....
C. vicina Consensus      GAAATCTTAA TACATCATTC TTTGACCCAG CAGGAGGAGG AGACCCAATC TTGT
C. vicina Wimbledon 1    .......... .......... .....T.... .......... .......... ....
C. vicina Wimbledon 2    .......... .......... .....T.... .......... .......... ....
C. vicina Wimbledon 3    .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 4    .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 5    .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 6    .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 7    .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 8    .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 9    .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 10   .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 11   .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 12   .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 13   .......... .......... .......... .......... .......... ....
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C. vicina Wimbledon 14          .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 15          .......... .......... .......... .......... .......... ....
C. vicina Wimbledon 16          .......... .......... .......... .......... .......... ....
C. vicina Pontypridd 1          .......... .......... .......... .......... .......... ....
C. vicina Pontypridd 2          .......... .......... .......... .......... .......... ....
C. vicina Nottingham 1          .......... .......... .......... .......... .......... ....
C. vicina Nottingham 2          .......... .......... .......... .......... .......... ....
C. vicina Nottingham 3          .......... .......... .......... .......... .......... ....
C. vicina Nottingham 4          .......... .......... .......... .......... .......... ....
C. vicina Nottingham 5          .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 1          .......... .......... .....T.... .......... .......... ....
C. vicina Cheltenham 2          .......... .......... .....T.... .......... .......... ....
C. vicina Cheltenham 3          .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 4          .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 5          .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 6          .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 7          .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 8          .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 9          .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 10         .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 11         .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 12         .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 13         .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 14         .......... .......... .......... .......... .......... ....
C. vicina Cheltenham 15         .......... ....
C. vicina Cheltenham 16         .......... .......... .......... .......... .......... ....
C. vicina Waterloo 1            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 2            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 3            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 4            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 5            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 6            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 7            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 8            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 9            .......... .......... .......... .......... .......... ....
C. vicina Waterloo 10           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 11           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 12           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 13           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 14           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 15           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 16           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 17           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 18           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 19           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 20           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 21           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 22           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 23           .......... .......... .......... .......... .......... ....
C. vicina Waterloo 24           .......... .......... .......... .......... .......... ....
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C. vicina Waterloo 25 .....
C. vicina Waterloo 26 .....
C. vicina Waterloo 27 .....
C. vicina Waterloo 28 .....
C. vicina Waterloo 29 .....
C. vicina Waterloo 30 .....
C. vicina Waterloo 31 .....
C. vicina Waterloo 32 .....
C. vicina Waterloo 33 .....
C. vicina Nonsuch Park 1 .....
C. vicina Nonsuch Park 2 .....
C. vicina Nonsuch Park 3 .....
C. vicina Nonsuch Park 4 .....
C. vicina Greenwich 1 .....
C. vicina Greenwich 2 ..
C. vicina Greenwich 3 .....
C. vicina Greenwich 4 .....
C. vicina Greenwich 5 .....
C. vicina Greenwich 6 .....
C. vicina Snaresbrook 1 .....
C. vicina Snaresbrook 2 .....
C. vicina Snaresbrook 3 .....
C. vicina Stanmore 1 .....
C. vicina Stanmore 2 .....
C. vicina Stanmore 3 .....
C. vicina Stanmore 4 .....
C. vicina Stanmore 5 .....
C. vicina Stanmore 6 .....
C. vicina York 1 .....
C. vicina York 2 .....
C. vicina York 3 .....
C. vicina York 4 .....
C. vicina York 5 .....
C. vicina Keswick 1 .....
C. vicina Keswick 2 .....
C. vicina Keswick 3 .....
C. vicina Keswick 4 .....
C. vicina Keswick 5 .....
C. vicina Great Yarmouth 1 .....
C. vicina Great Yarmouth 2 .....
C. vicina Great Yarmouth 3 .....
C. vicina Great Yarmouth 4 .....
C. vicina Great Yarmouth 5 .....
C. vicina Derby 1 .....
C. vicina Mitcham Junction 1 .....
C. vicina Mitcham Junction 2 .....
C. vicina Mitcham Junction 3 .....
C. vicina Mitcham Junction 4 .....
C. vicina Larva 1 .....
C. vicina Larva 2 .....

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C. vicina Larva 3                .......... .......... .......... .......... .......... ....
C. vicina Larva 4                .......... .......... .......... .......... .......... ....
C. vicina Larva 5                .......... .......... .....T.... .......... .......... ....
C. vicina Larva 6                .......... .......... .......... .......... .......... ....
C. vicina Larva 7                .......... .......... .......... .......... .......... ....
C. vicina Larva 8                .......... .......... .......... .......... .......... ....
C. vomitoria Consensus           .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria WimbledonCommon1    .......... ...T...... .......... .......... ...T.....T
C. vomitoria WimbledonCommon2
C. vomitoria WimbledonCommon3    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Hamstead Heath 1    .......... ...T...... .......... .......... ..
C. vomitoria Hamstead Heath 2    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Hamstead Heath 3    .......... ...T.... . .......... .......... ...T.....T ..A.
C. vomitoria Hamstead Heath 4    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria York 1              .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Nonsuch Park 1      .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Nonsuch Park 2      .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Nonsuch Park 3      .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Nonsuch Park 4      .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Nonsuch Park 5      .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Nonsuch Park 6      .......... ...T...... .......
C. vomitoria Nonsuch Park 7      .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Nonsuch Park 8      .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Keswick 1           .......... ...T...... ..
C. vomitoria Keswick 2           .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Keswick 3           .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Keswick 4           .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Keswick 5           .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Petts Wood 1        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Petts Wood 2        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Petts Wood 3        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Boxhill 1           .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Boxhill 2           .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria East Sheen 1        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria East Sheen 2        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria East Sheen 3        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria East Sheen 4        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria East Sheen 5        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Mitcham Junction1   .......... ...T...... ..
C. vomitoria Mitcham Junction2   .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Mitcham Junction3   .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Mitcham Junction4   .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Mitcham Junction5   .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Stanmore 1          .......... ...T...... ..
C. vomitoria Stanmore 2          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Stanmore 3          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Stanmore 4          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Cheltenham 1        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Cheltenham 2        .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 1                   .......... ...T...... .......... .......... ...T.....T ..A.
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C. vomitoria 2     .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 3
C. vomitoria 4     .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 5     .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 6     .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 7     .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 8     .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 9     .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 10    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 11    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 12    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 13    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 14    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 15    .......... ...T...... .....T.... .......... ...T.....T ..A.
C. vomitoria 16    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 17    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 18    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 19    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 20    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 21    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 22    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 23    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 24    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 25    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 26    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 27    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 28    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 29    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 30    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 31    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 32    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 33    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 34    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 35    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 36    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 37    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 38    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 39    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 40    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 41    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 42    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 43    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 44    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 45    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 46    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 47    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 48    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 49    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 50    .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria 51    .......... ...T...... .......... .......... ...T.....T ..A.
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C. vomitoria Larva 1          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 2          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 3          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 4          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 5          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 6          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 7          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 8          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 9          .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 10         .......... ...T...... .......... .......... ...T.....T ..A.
C. vomitoria Larva 11         .......... ...T...... .......... .......... ...T.....T ..A.
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Appendix III.

Insect Mitochondrial Genetic Code based upon Clary and Wolstenholme (1985). See Abbreviations for one letter and three letter codes. Grey shading indicates difference to standard genetic code.

First Codon Position	Second Codon position				Third Codon Position
	A	G	C	T	
A	Lys (K)	Ser (S)	Thr (T)	Met (M)	A
	Lys (K)	Ser (S)	Thr (T)	Met (M)	G
	Asn (N)	Ser (S)	Thr (T)	Ile (I)	C
	Asn (N)	Ser (S)	Thr (T)	Ile (I)	T
G	Glu (E)	Gly (G)	Ala (A)	Val (V)	A
	Glu (E)	Gly (G)	Ala (A)	Val (V)	G
	Asp (D)	Gly (G)	Ala (A)	Val (V)	C
	Asp (D)	Gly (G)	Ala (A)	Val (V)	T
C	Gln (Q)	Arg (R)	Pro (P)	Leu (L)	A
	Gln (Q)	Arg (R)	Pro (P)	Leu (L)	G
	His (H)	Arg (R)	Pro (P)	Leu (L)	C
	His (H)	Arg (R)	Pro (P)	Leu (L)	T
T	TER	Trp (W)	Ser (S)	Leu (L)	A
	TER	Trp (W)	Ser (S)	Leu (L)	G
	Tyr (Y)	Cys (C)	Ser (S)	Phe (F)	C
	Tyr (Y)	Cys (C)	Ser (S)	Phe (F)	T

Appendix IV.

Alignment of COI partial gene region for Calliphoridae samples deposited in GenBank. Dot indicates similar nucleotide to first *C.vicina* sequence.

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                                          10        20        30        40        50        60        70        80        90       100       110       120
                                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Calliphora vicina  gi|44238800  CCTTCCCTCG GATAAACAAT ATAAGTTTCT GACTTTTACC TCCTGCATTA ACTTTACTAT TAGTAAGTAG TATAGTAGAA AACGGAGCTG GAACAGGATG AACTGTTTAC CCACCTTTAT
Calliphora vicina  gi|21727797  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409264  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409266  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409262  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409260  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409258  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409256  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409254  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vomitoria gi|442388  .A........ A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
Calliphora vomitoria gi|294092  .A........ A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
Calliphora vomitoria gi|294092  .A........ A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
Calliphora vomitoria gi|294092  .A........ A.....T... .......... .......... .......... .......... .......... .......... .......... ....T..... .........T ..........
Calliphora vomitoria gi|294092  .A........ A.....T... .......... .......... .......... .....T.... .......... .......... .......... ....T..... .........T ..........
Calliphora vomitoria gi|294092  .A........ A.....T... .......... .......... .......... .....T.... .......... .......... .......... ....T..... .........T ..........
Calliphora vomitoria gi|294092  .A........ A.....T... .......... .......... .......... .....T.... .......... .......... .......... ....T..... .........T ..........
Calliphora vomitoria gi|294092  .A........ A.....T... .......... .......... .......... .....T.... .......... .......... .......... ....T..... .........T ..........
Eucalliphora latifrons gi|1132  .......... A.....T... .......T. .......... .......... .......... .......... .......... .......... .......... .....C....
Lucilia adisoemartoi gi|198795  .A........ AT....T... .....A..T. .......... .......... .....T.... .......... .......... .......... ....T..... ...A...... ..........
Lucilia ampullacea gi|29409234  .......... AT....T... .....A..T. .......... .......C.. .....T.... .......... .......... .......... .......... ...A...... ..C.......
Lucilia ampullacea gi|29409232  .......... AT....T... .....A..T. .......... .......C.. .....T.... .......... .......... .......... .......... ...A...... ..C.......
Lucilia ampullacea gi|29409230  .......... AT....T... .....A..T. .......... .......C.. .....T.... .......... .......... .......... .......... ...A...... ..C.......
Lucilia ampullacea gi|29409228  .......... AT....T... .....A..T. .......... .......C.. .....T.... .......... .......... .......... .......... ...A...... ..C.......
Lucilia ampullacea gi|29409226  .......... AT....T... .....A..T. .......... .......C.. .....T.... .......... .......... .......... .......... ...A...... ..C.......
Lucilia bazini gi|33667901|     .A........ A.....T... .....A..T. ....C..... .......... .....T.G.. .......... .......... .......... .......... ...A...... ..C.......
Lucilia caesar gi|29409218|     .A........ A.....T... .....A..T. .......... .......... .....T.... .......... .......... .......... .......... ...A...... ..T.......
Lucilia caesar gi|29409216|     .A........ A.....T... .....A..T. .......... .......... .....T.... .......... .......... .......... .......... ...A...... ..T.......
Lucilia caesar gi|21727823|     .A........ A.....T... .....A..T. .......... .......... .....T.... .......... .......... .......... .......... ...A...... ..T.......
Lucilia caesar gi|29409224      .A........ A.....T... .....A..T. .......... .......... .....T.... .......... .......... .......... .......... ...A...... ..T.......
Lucilia caesar gi|29409222|     .A........ A.....T... .....A..T. .......... .......... .....T.... .......... .......... .......... .......... ...A...... ..T.......
Lucilia caesar gi|29409220      .A........ A.....T... .....A..T. .......... .......... .....T.... .......... .......... .......... .......... ...A...... ..T.......
Lucilia cuprina gi|21727832|    .A........ A.....T... .......T. .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|21727835|    .A........ A.....T... .......T. .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|21727826|    .A........ A.....T... .......T. .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T..CC...
Lucilia cuprina gi|4204934      .A........ A.....T... .......TG .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T..CC...
Lucilia cuprina gi|4204926|     .A........ A.....T... .......TG .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T..CC...
Lucilia cuprina gi|4204932|     .A........ A.....T... .......TG .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T..CC...
Lucilia cuprina gi|4204940|     .A........ A.....T... .......TG .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|4204936      .A........ A.....T... .......TG .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|4204930|     .A........ A.....T... .......TG .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|4204928|     .A........ A.....T... .......TG .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|4204938      .A........ A.....T... .......TG .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|21727844|    .A........ A.....T... .......T. .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|21727838|    .A........ A.....T... .......T. .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|39652363                                    .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|39652343                                    .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|39652341                                    .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia cuprina gi|21386902|    .A........ A.....T... .......T. .......... .......... .....T.... ....T..... .......... .......... .......... ...A...... ..T..CC...
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Lucilia cuprina gi|21727829|          .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T..CC...
Lucilia cuprina gi|21727841|          .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia hainanensis gi|3366790        .A........ A.....T... .....A..T. .....C.... .......... ......T... .......... .......... .......... .......... ...A...... ..T...C...
Lucilia illustris gi|36788203         .A........ A.....T... .....A..T. .......... .......... ......T... .......... .......... .......... .......... ...G...... ..........
Lucilia illustris gi|29409250|        .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia illustris gi|29409244|        .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia illustris gi|29409236         .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia illustris gi|29409248|        .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia illustris gi|29409252|        .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia illustris gi|29409242|        .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia illustris gi|29409240         .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia illustris gi|29409238|        .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia illustris gi|29409246|        .A........ A.....T... .....A..T. .......... .......... .......... .......... .......... .......... .......... ...A...... ..T.......
Lucilia porphyrina gi|21386904        .......... AT....T... .....A..T. .......... .......C.. ......T... .......... .......... .......... .......... ...A...... ..C.......
Lucilia porphyrina gi|19879590        .......... AT....T... .....A..T. .......... .......... ......T... .......... .......... .......... .G........ ...A...... ..T.......
Lucilia sericata gi|21727847          .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|21727859|         .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|21727850|         .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......G.. ...A...... ..T...C...
Lucilia sericata gi|21727853          .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|21727856|         .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|21727862|         .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|29409284          .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|29409282|         .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|29409286|         .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|29409290          .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|39652365                                           .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|39652355                                           .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|39652325                                           .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|39652323                                           .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|29409288|         .A........ A.....T... ........T. .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|39652303                                           .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Lucilia sericata gi|39652337                                           .......... .......... ......T... ....T..... .......... .......... .......... ...A...... ..T...C...
Chrysomya albiceps gi|39652345                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|39652339                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|39652321                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|39652315                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|39652309                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|3659514|        .T.....A.. A.....T... .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|39652367                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|39652353                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|39652317                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya albiceps gi|39652335                                         .......... .......... .......... .......... .......... ..T....... .......... .........T ..........
Chrysomya bezziana gi|11321181        .T.....A.. A.....T... .......... .......... .......... ......T.G. .......... .......... ..T....... .......... .......... ..........
Chrysomya inclinata gi|3965235                                         .......... .......... ...C..T... .......... .......... ..T..G.... .......... .......... ..........
Chrysomya marginalis gi|396523                                         .......... ......T... ...C...... .......... .......... ..T....... .......... .......... ..........
Chrysomya marginalis gi|396523                                         .......... ......T... ...C...... .......... .......... ..T....... .......... .......... ..........
Chrysomya marginalis gi|396523                                         .......... ......T... ...C...... .......... .......... ..T....... .......... .......... ..........
Chrysomya marginalis gi|396523                                         .......... ......T... ...C...... .......... .......... ..T....... .......... .......... ..........
Chrysomya marginalis gi|396523                                         .......... ......T... ...C...... .......... .......... ..T....... .......... .......... ..........
Chrysomya marginalis gi|396523                                         .......... ......T... ...C...... .......... .......... ..T....... .......... .......... ..........
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Chrysomya megacephala gi|39652
Chrysomya megacephala gi|39652                                                
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Lucilia ampullacea gi|29409234  .A..... ..... ..... ..... ..... C.T..T.... ..... ..... ..G..... ..... .....C... .....
Lucilia ampullacea gi|29409232  .A..... ..... ..... ..... ..... C.T..T.... ..... ..... ..G..... ..... .....C... .....
Lucilia ampullacea gi|29409230  .A..... ..... ..... ..... ..... C.T..T.... ..... ..... ..G..... ..... .....C... .....
Lucilia ampullacea gi|29409228  .A..... ..... ..... ..... ..... C.T..T.... ..... ..... ..G..... ..... .....C... .....
Lucilia ampullacea gi|29409226  .A..... ..... ..... ..... ..... C.T..T.... ..... ..... ..G..... ..... .....C... .....
Lucilia bazini gi|33667901|     .A..... ..... ..... ..... .....C C.T..T.... ..... ..... ..... ..... ..... .....A. .....
Lucilia caesar gi|29409218|     .A..... ..... ..... ..... ..... C.T..T.... .....C.. A..... ..G..... ..... ..... .....
Lucilia caesar gi|29409216|     .A..... ..... ..... ..... ..... C.T..T.... .....C.. A..... ..G..... ..... ..... .....
Lucilia caesar gi|21727823|     .A..... ..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia caesar gi|29409224      .A..... ..... ..... ..... ..... C.T..T.... .....C.. A..... ..G..... ..... ..... .....
Lucilia caesar gi|29409222|     .A..... ..... ..... ..... ..... C.T..T.... .....C.. A..... ..G..... ..... ..... .....
Lucilia caesar gi|29409220      .A..... ..... ..... ..... ..... C.T..T.... .....C.. A..... ..G..... ..... ..... .....
Lucilia cuprina gi|21727832|    ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|21727835|    ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|21727826|    ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia cuprina gi|4204934      ..... T..... ..... ..... .....C... C.T..T.... ..... .....G ..... ..... ..... .....
Lucilia cuprina gi|4204926|     ..... T..... ..... ..... .....C... C.T..T.... ..... .....G ..... ..... ..... .....
Lucilia cuprina gi|4204932|     ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia cuprina gi|4204940|     ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|4204936      ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|4204930|     ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|4204928|     ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|4204938      ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|21727844|    ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|21727838|    ..... T.....C... ..... ..... .....C... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|39652363     ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|39652343     ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|39652341     ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia cuprina gi|21386902|    ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia cuprina gi|21727829|    ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia cuprina gi|21727841|    ..... T.....C... ..... ..... ..... C.T..T.... ..... C..... ..... ..... ..... .....
Lucilia hainanensis gi|3366790  .A..C..... T..... .....C.... ..... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia illustris gi|36788203   .A..... ..... ..... ..... ..... C.T..T.... .....C.. A..... ..G..... ..... ..... .....
Lucilia illustris gi|29409250|  .A..... ..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia illustris gi|29409244|  .A..... T..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia illustris gi|29409236   .A..... T..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia illustris gi|29409248|  .A..... ..... ..... ..... ..... C.T..T.... ..... A..... ..G..... ..... ..... .....
Lucilia illustris gi|29409252|  .A..... ..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia illustris gi|29409242|  .A..... ..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia illustris gi|29409240   .A..... ..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia illustris gi|29409238|  .A..... ..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia illustris gi|29409246|  .A..... T..... ..... ..... ..... C.T..T.... ..... A..... ..... ..... ..... ..... .....
Lucilia porphyrina gi|21386904  .A..... T..... ..... ..... ..... C.T..T.... ..... ..... ..... ..... ..... .....G....
Lucilia porphyrina gi|19879590  .A..... ..... ..... ..... ..... C.T..T.... ..... ..... ..... ..... .....C... .....
Lucilia sericata gi|21727847    ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia sericata gi|21727859|   ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia sericata gi|21727850|   ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia sericata gi|21727853    ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia sericata gi|21727856|   ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
Lucilia sericata gi|21727862|   ..... T..... ..... ..... .....C... C.T..T.... ..... ..... ..... ..... ..... .....
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Lucilia sericata gi|29409284          .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|29409282|         .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|29409286|         .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|29409290          .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|39652365          .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|39652355          .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|39652325          .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|39652323          .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|29409288|         .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|39652303          .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Lucilia sericata gi|39652337          .......... T......... .......... .......... ......C... C.T..T.... .......... .......... .......... .......... .......... ..........
Chrysomya albiceps gi|39652345        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|39652339        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|39652321        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|39652315        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|39652309        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|3659514|        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|39652367        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|39652353        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|39652317        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya albiceps gi|39652335        .A........ T........T .....A..A. .......... .......... .......... .T........ A......... .......... .......... A..T...... ..........
Chrysomya bezziana gi|11321181        .......... T.....C... .....A..A. .C........ ......C... .....T.... .......... C......C.. .......... .......... A..T..A... ..........
Chrysomya inclinata gi|3965235        .A........ T.....C..T .....A..A. .......... ......C... .....T.... .T..G..... .......... .......... .......... A..T..A... ..........
Chrysomya marginalis gi|396523        .A........ T..C...... ..T..A..A. .......... ......C..A C....T.... .T........ C......... .......... .......... A..T..A... ..........
Chrysomya marginalis gi|396523        .A........ T..C...... ..T..A..A. .......... ......C..A C....T.... .T........ C......... .......... .......... A..T..A... ..........
Chrysomya marginalis gi|396523        .A........ T..C...... ..T..A..A. .......... ......C..A C....T.... .T........ C......... .......... .......... A..T..A... ..........
Chrysomya marginalis gi|396523        .A........ T..C...... ..T..A..A. .......... ......C..A C....T.... .T........ C......... .......... .......... A..T..A... ..........
Chrysomya megacephala gi|39652        .......... T......... .....A..A. .......... ......C... .......... .......... .......... .......... .......... A..T..A... ..........
Chrysomya megacephala gi|39652        .......... T......... .....A..A. .......... ......C... .......... .......... .......... .......... .......... A..T..A... ..........
Chrysomya megacephala gi|20453        .......... T......... .....A..A. .......... ......C... .......... .......... ......C... .......... .......... A..T..A... ..........
Chrysomya megacephala gi|39652        .......... T......... .....A..A. .......... ......C... .......... .......... .......... .......... .......... A..T..A... ..........
Chrysomya megacephala gi|11321        .......... T......... .....A..A. .......... ......C... .......... .......... .......... .......... .......... A..T..A... ..........
Chrysomya megacephala gi|39652        .......... T......... .....A..A. .......... ......C... .......... .......... .......... .......... .......... A..T..A... ..........
Chrysomya megacephala gi|39652        .......... T......... .....A..A. .......... ......C... .......... .......... .......... .......... .......... A..T..A... ..........
Chrysomya megacephala gi|39652        .......... T......... .....A..A. .......... ......C... .......... .......... .......... .......... .......... A..T..A... ..........
Chrysomya norrisi gi|11321193|        .A........ T.....C... ........A. .......... .......... C.T....... .......... .......... .......... ....C..... ...T..A... ..........
Chrysomya pinguis gi|20453018|        .......... T....T.... .....A..A. .......... ......C... .....T.... .......... .......... .......... .C..C..... A..T..A... ..........
Chrysomya putoria gi|39652347         .A........ T..C.....T ..T..A..A. .......... ......C... .....T.... .C........ .......... .......... .......... A..T..A... ..........
Chrysomya putoria gi|39652307         .A........ T..C.....T ..T..A..A. .......... ......C... .....T.... .C........ .......... .......... .......... A..T..A... ..........
Chrysomya putoria gi|11321199|        .A........ T..C.....T ..T..A..A. .......... ......C... .....T.... .C........ .......... .......... .......... A..T..A... ..........
Chrysomya putoria gi|39652357         .A........ T..C.....T ..T..A..A. .......... ......C... .....T.... .C........ .......... .......... .......... A..T..A... ..........
Chrysomya putoria gi|39652299         .A........ T..C.....T ..T..A..A. .......... ......C... .....T.... .C........ .......... .......... .......... A..T..A... ..........
Chrysomya rufifacies gi|365951        .A........ T..A.....T .....A..A. .......... .......... .......... .T........ A......... ..G..C.... .......... .......... A..T...... ..........
Chrysomya rufifacies gi|396523        .A........ T..A.....T .....A..A. .......... .......... .......... .T........ A......... ..G..C.... .......... .......... A..T...... ..........
Chrysomya rufifacies gi|396522        .A........ T..A.....T .....A..A. .......... .......... .......... .T........ A......... ..G..C.... .......... .......... A..T...... ..........
Chrysomya semimetallica gi|113        .......... .......... .....A..A. .......... ......C... C.T..T.... .C........ C......... .....A.... .......... .......... A.....A... ..........
Chrysomya varipes gi|39652375         .A........ T.....C... ........A. .......... ......C... .....T.... .......... .......... .......... .......... .......... A..T..A... ..........
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Chrysomya varipes gi|39652373   .A..... T.....C... .....A. ..... .....C... .....T.... ..... ..... ..... ..... A..T..A... .....
Chrysomya varipes gi|39652371   .A..... T.....C... .....A. ..... .....C... .....T.... ..... ..... ..... ..... A..T..A... .....
Chrysomya varipes gi|11321205|  .A..... T.....C... .....A. ..... .....C... .....T.... ..... ..... ..... ..... A..T..A... .....
Cochliomyia macellaria gi|1132  ..... T.....C... .....A. .....C... .....C... C.T..T.... .C..... A..... .....A.... ....C..... A..T..A... .....
Phormia regina gi|8272632       .A..... T..... .....A... .....C... .....C... C.T..... ..... ..... ..... ..... ....C..... A..T..A... .....
Phormia regina gi|11321187|     .A..... T..... .....A... .....C... .....C... C.T..... ..... ..... ..... ..... ....C..... A..T..A... .....
Drosophila yakuba               ....AGG... .....T ..... .A..... ..... C.T..T.... .T..... ..... ..... ..... ..... G..T..A... .....

                                        250       260       270       280       290       300       310       320       330       340       350       360
                                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Calliphora vicina  gi|44238800  CAACAGGAAT TACATTCGAC CGAATACCAT TATTTGTTTG ATCTGTAGTA ATTACAGCTT TATTACTTTT ATTATCTTTA CCAGTATTAG CAGGTGCTAT TACTATATTA TTAACAGATC
Calliphora vicina  gi|21727797  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409264  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409266  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409262  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409260  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409258  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409256  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vicina  gi|29409254  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Calliphora vomitoria gi|442388  .......T.. ...C...... .......... .......... ...A...... ........C. .......... .......... .......... .......... .......... ..........
Calliphora vomitoria gi|294092  .......T.. ...C...... .......... .......... ...A...... ........C. .......... .......... .......... ....A..... .......... ..........
Calliphora vomitoria gi|294092  .......T.. ...C...... .......... .......... ...A...... ........C. .......... .......... .......... ....A..... .......... ..........
Calliphora vomitoria gi|294092  .......T.. ...C...... .......... .......... ...A...... ........C. .......... .......... .......... ....A..... .......... ..........
Calliphora vomitoria gi|294092  .......T.. ...C...... .......... .......... ...A...... ........C. .......... .......... .......... ....A..... .......... ..........
Calliphora vomitoria gi|294092  .......T.. ...C...... .......... .......... ...A...... ........C. .......... .......... .......... ....A..... .......... ..........
Calliphora vomitoria gi|294092  .......T.. ...C...... .......... .......... ...A...... ........C. .......... .......... .......... ....A..... .......... ..........
Eucalliphora latifrons gi|1132  .......... ...T..T... ........TC .T..... .......... .......... .......... .......... .......... .C..... .......C.. ..........
Lucilia adisoemartoi gi|198795  .......... ...T..T... ........T. ....C..... ...A...... .......... .......... .......... ......AC.. .......... .C..A..... C..A...C.T .....T..C.
Lucilia ampullacea gi|29409234  .......... ...T..T... ........T. .......... ...A..T... .........C .......... ......A... .......... ....A..... ...A...C.T .....T..C.
Lucilia ampullacea gi|29409232  .......... ...T..T... ........T. .......... ...A..T... .........C .......... ......A... .......... ....A..... ...A...C.T .....T..C.
Lucilia ampullacea gi|29409230  .......... ...T..T... ........T. .......... ...A..T... .........C .......... ......A... .......... ....A..... ...A...C.T .....T..C.
Lucilia ampullacea gi|29409228  .......... ...T..T... ........T. .......... ...A..T... .........C .......... ......A... .......... ....A..... ...A...C.T .....T..C.
Lucilia ampullacea gi|29409226  .......... ...T..T... ........T. .......... ...A..T... .........C .......... ......A... .......... ....A..... ...A...C.T .....T..C.
Lucilia bazini gi|33667901|     .......... ......T..T ........T. .......... .......... .......... ........C. ......A... .......... .C..A..... C..A..... .....T....
Lucilia caesar gi|29409218|     .......... ...T..T... ........T. ....C..... ...A...... .......... .......... ......A... .......... .T..A..... .......C.T .....T..C.
Lucilia caesar gi|29409216|     .......... ...T..T... ........T. ....C..... ...A...... .......... .......... ......A... .......... .T..A..... .......C.T .....T..C.
Lucilia caesar gi|21727823|     .......G.. ...T..T..T .....G..T. ....C..... ...A...... .......... .......... ......A... .......... .T..A..... .......C.T .....T....
Lucilia caesar gi|29409224      .......... ...T..T... ........T. ....C..... ...A...... .......... .......... ......A... .......... .T..A..... .......C.T .....T..C.
Lucilia caesar gi|29409222|     .......... ...T..T... ........T. ....C..... ...A...... .......... .......... ......A... .......... .T..A..... .......C.T .....T..C.
Lucilia caesar gi|29409220      .......... ...T..T... ........T. ....C..... ...A...... .......... .......... ......A... .......... .T..A..... .......C.T .....T..C.
Lucilia cuprina gi|21727832|    .......... ...T..T..T ........T. .......G.. ...A...... .......... .......... ......A... .......... .T..A..... ...A...C.T ..........
Lucilia cuprina gi|21727835|    .......... ...T..T..T ........T. .......G.. ...A...... .......... .......... ......A... .......... .T..A..... ...A...C.T ..........
Lucilia cuprina gi|21727826|    .......... ...T..T..T ........T. .......... ...A...... .......... .....G..... ......A... .......... ....A..... ...A...C.T ........C.
Lucilia cuprina gi|4204934      .......... ...T..T..T ........T. .......... ...A...... .......... .....G..... ......A... ...C...... ....A..... ...A...C.T ........C.
Lucilia cuprina gi|4204926|     .......... ...T..T..T ........T. .......... ...A...... .......... .....G..... ......A... .......... ....A..... ...A...C.T ........C.
Lucilia cuprina gi|4204932|     .......... ...T..T..T ........T. .......... ...A...... .......... .....G..... ......A... .......... ....A..... ...A...C.T ........C.
Lucilia cuprina gi|4204940|     .......... ...T..T..T ........T. .......G.. ...A...... .......... .......... ......A... .......... .T..A..... ...A...C.T ..........
Lucilia cuprina gi|4204936      .......... ...T..T..T ........T. .......G.. ...A...... .......... .......... ......A... .......... .T..A..... ...A...C.T ..........
Lucilia cuprina gi|4204930|     .......... ...T..T..T ........T. .......G.. ...AA..A.. .......... .......... ......A... .......... .T..A..... ...A...C.T ..........
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Lucilia cuprina gi|4204928|                ..........  ...T..T..T  ........T.  .......G..  ...AA..A..  ..........  ..........  ......A...  ..........  .T..A.....  ...A...C.T  ..........
Lucilia cuprina gi|4204938                 ..........  ...T..T..T  ........T.  .......G..  ...A......  ..........  ..........  ......A...  .........C  .T..A.....  ...A...C.T  ..........
Lucilia cuprina gi|21727844|               ..........  ...T..T..T  ........T.  .......A..  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  ...A...C.T  ..........
Lucilia cuprina gi|21727838|               ..........  ...T..T..T  ........T.  .......A..  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  ...A...C.T  ..........
Lucilia cuprina gi|39652363                ..........  ...T..T..T  ........T.  .......G..  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  ...A...C.T  ..........
Lucilia cuprina gi|39652343                ..........  ...T..T..T  ........T.  .......G..  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  ...A...C.T  ..........
Lucilia cuprina gi|39652341                ..........  ...T..T..T  ........T.  .......G..  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  ...A...C.T  ..........
Lucilia cuprina gi|21386902|               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ....G.....  .C....A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia cuprina gi|21727829|               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ....G.....  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia cuprina gi|21727841|               ..........  ...T..T..T  ........T.  .......A..  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  ...A...C.T  ..........
Lucilia hainanensis gi|3366790             ..........  ...T..T..T  ........T.  ..........  ..........  ..........  ..........  .C....A...  ..........  ....A.....  ...A...C.T  .....T..C.
Lucilia illustris gi|36788203              ..........  ...T..T...  ........T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T..C.
Lucilia illustris gi|29409250|             .......G..  ...T..T..T  .....G..T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T....
Lucilia illustris gi|29409244|             .......G..  ...T..T..T  .....G..T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T....
Lucilia illustris gi|29409236              .......G..  ...T..T..T  .....G..T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T....
Lucilia illustris gi|29409248|             .......G..  ...T..T..T  ........T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T....
Lucilia illustris gi|29409252|             .......G..  ...T..T..T  ........T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T..C.
Lucilia illustris gi|29409242|             .......G..  ...T..T..T  .....G..T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T....
Lucilia illustris gi|29409240              .......G..  ...T..T..T  .....G..T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T....
Lucilia illustris gi|29409238|             .......G..  ...T..T..T  .....G..T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T....
Lucilia illustris gi|29409246|             .......G..  ...T..T..T  .....G..T.  ....C.....  ...A......  ..........  ..........  ......A...  ..........  .T..A.....  .......C.T  .....T....
Lucilia porphyrina gi|21386904             ..........  ...T..T...  ........T.  ..........  ...A..T...  ..........  .........C  ..........  ......A...  ..........  ....A.....  .....C.T  .....T..C.
Lucilia porphyrina gi|19879590             ..........  ...T..T...  ........T.  ..........  ...A..T...  ..........  .........C  ..........  ......A...  ..........  ....A.....  ...A...C.T  .....T..C.
Lucilia sericata gi|21727847               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|21727859|              ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|21727850|              ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|21727853               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|21727856|              ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|21727862|              ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|29409284               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|29409282|              ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|29409286|              ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|29409290               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|39652365               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|39652355               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|39652325               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|39652323               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|29409288|              ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|39652303               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Lucilia sericata gi|39652337               ..........  ...T..T..T  ........T.  ..........  ...A......  ..........  ..........  ..........  ......A...  ..........  ....A.....  ...A...C.T  ........C.
Chrysomya albiceps gi|39652345             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|39652339             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|39652321             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|39652315             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|39652309             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|3659514|             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|39652367             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|39652353             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|39652317             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
Chrysomya albiceps gi|39652335             .T........  C.....T..T  ........T.  ....C..A..  ..........  .....T...C  .TC.TT.A..  ......A...  ..........  .C.....A..  ..........  .....T....
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Chrysomya bezziana gi|11321181  .T..... ..... .T..T .....T. ....C..A.. .....T ..... .....T... ..... ..... .T..A..... ......G... .....T..C.
Chrysomya inclinata gi|3965235  .T..... ..... .T... ..... ....C..... .....T ..... .....T...C ..... ..... .C..A..... ..... .....C. .....C.
Chrysomya marginalis gi|396523  .T..... ..... .T..T .....T. ..... ..... ..... .....T.... ..... ..... G..... ..... ....A..... .....C.. .....T....
Chrysomya marginalis gi|396523  .T..... ..... .T..T .....T. ..... ..... ..... .....T.... ..... ..... G..... ..... ....A..... .....C.. .....T....
Chrysomya marginalis gi|396523  .T..... ..... .T..T .....T. ..... ..... ..... .....T.... ..... ..... G..... ..... ....A..... .....C.. .....T....
Chrysomya marginalis gi|396523  .T..... ..... .T..T .....T. ..... ..... ..... .....T.... ..... ..... G..... ..... ....A..... .....C.. .....T....
Chrysomya marginalis gi|396523  .T..... ..... .T..T .....T. ..... ..... ..... .....T.... ..... ..... G..... ..... ....A..... .....C.. .....T....
Chrysomya megacephala gi|39652  .T..... ..... .T..T .....T. .....A.. ..... .....T.... ..... ..... G..... ..... ....A..... .....C.. .....T....
Chrysomya megacephala gi|39652  .T..... ..... .T..T .....T. .....A.. .....T ....T...C .....T.A.. ..... ..... .T..A..... ..... .....T..C.
Chrysomya megacephala gi|20453  .T..... ..... .T..T .....T. .....A.. .....T ....T...C .....T.A.. ..... ..... .T..A..... ..... .....T..C.
Chrysomya megacephala gi|39652  .T..... ..... .T..T .....T. .....A.. .....T ....T...C .....T.A.. ..... ..... .T..A..... ..... .....T..C.
Chrysomya megacephala gi|11321  .T..... ..... .T..T .....T. .....A.. .....T ....T...C .....T.A.. ..... ..... .T..A..... ..... .....T..C.
Chrysomya megacephala gi|39652  .T..... ..... .T..T .....T. .....A.. .....T ....T...C .....T.A.. ..... ..... .T..A..... ..... .....T..C.
Chrysomya megacephala gi|39652  .T..... ..... .T..T .....T. .....A.. .....T ....T...C .....T.A.. ..... ..... .T..A..... ..... .....T..C.
Chrysomya megacephala gi|39652  .T..... ..... .T..T .....T. .....A.. .....T ....T...C .....T.A.. ..... ..... .T..A..... ..... .....T..C.
Chrysomya megacephala gi|39652  .T..... ..... .T..T .....T. .....A.. .....T ....T..CC .....T.A.. ..... ..... .T..A..... ..... .....T..C.
Chrysomya norrisi gi|11321193|  .T..... ..... .T... .....T. ..... ..... .....T ....T...C .....T.A.. ..... ..... .T..A..... ..... ..G..T....
Chrysomya pinguis gi|20453018|  .T..... ..... .T..T .....T. .....A.. ..... .....T ....T...C .....T.A.. ..... ..... .C..A..... ..... .....T..C.
Chrysomya putoria gi|39652347   .T..... ..... .T... ..... ....C..... ..... .....T ....T...C .....T.A.. ..... ..... ....A..... ..... .....T..C.
Chrysomya putoria gi|39652307   .T..... ..... .T... ..... ....C..... ..... .....T ....T...C .....T.A.. ..... ..... ....A..... ..... .....T..C.
Chrysomya putoria gi|11321199|  .T..... ..... .T... ..... ....C..... ..... .....T ....T...C .....T.A.. ..... ..... ....A..... ..... .....T....
Chrysomya putoria gi|39652357   .T..... ..... .T... ..... ....C..... ..... .....T ....T...C .....T.A.. ..... ..... ....A..... ..... .....T..C.
Chrysomya putoria gi|39652299   .T..... ..... .T... ..... ....C..... ..... .....T ....T...C .....T.A.. ..... ..... ....A..... ..... .....T..C.
Chrysomya rufifacies gi|365951  .T..... ..... .T..T .....T. .....A.. ..... .....T ....T...C .TC.TT.A.. ......A... ..... .....A.. ..... .....T....
Chrysomya rufifacies gi|396523  .T..... ..... .T..T .....T. .....A.. ..... .....T ....T...C .TC.TT.A.. ......A... ..... .....A.. ..... .....T....
Chrysomya rufifacies gi|396522  .T..... ..... .T..T .....T. .....A.. ..... .....T ....T...C .TC.TT.A.. ......A... ..... .....A.. ..... .....T....
Chrysomya semimetallica gi|113  ..... ..... .T..T .....T. ....C..... ..... .....T ....T...C .....T.A.. ......A... ..... ....A..... ..... .....T..C.
Chrysomya varipes gi|39652375   .T..... ..... .T... .....T. ....C..... ..... .....T ....T...C .TC.TT.A.. ..... ..... ....A..... .....C.. .....T....
Chrysomya varipes gi|39652373   .T..... ..... .T... .....T. ....C..... ..... .....T ....T...C .TC.T..A.. ..... ..... ....A..... .....C.. .....T....
Chrysomya varipes gi|39652371   .T..... ..... .T... .....T. ....C..... ..... .....T ....T...C .TC.T..A.. ..... .....G.... ....A..... .....C.. .....T....
Chrysomya varipes gi|113212051  .T..... ..... .T... .....T. ....C..... ..... .....T ....T...C .TC.T..A.. ..... ..... ....A..... .....C.. .....T....
Cochliomyia macellaria gi|1132  ..... ..... .....T ....G..T. ....C..A.. ...A..... ..... .....T ....T...C .T..... ..... ..... .....T.... .C..A..... .....C.T .....T....
Phormia regina gi|8272632       ....T..... ..... .T..T .....T. ..... ..... ..... .....T ....T...C ..... ..... ..... ..T..... .C..... ..... ..... .....T....
Phormia regina gi|11321187|     ....T..... ..... .T..T .....T. ..... ..... ..... .....T ....T...C ..... ..... .....G ..T..... .C..... ..... ..... .....T....
Drosophila yakuba               ....T..... ..... .A... .....T. .....A.. ...A..... .....T ....T.... ..... ..... .C..... ..... .....TC.T. .C..A..... ..... ..... .....C.

                                          370         380         390         400         410
                                ....|....| ....|....| ....|....| ....|....| ....|....| ....
Calliphora vicina  gi|44238800  GAAATCTTAA TACATCATTC TTTGACCCAG CAGGAGGAGG AGACCCAATC TTGT
Calliphora vicina  gi|21727797  .......... .......... .......... .......... .......... ....
Calliphora vicina  gi|29409264  .......... .......... .....T.... .......... .......... ....
Calliphora vicina  gi|29409266  .......... .......... .....T.... .......... .......... ....
Calliphora vicina  gi|29409262  .......... .......... .....T.... .......... .......... ....
Calliphora vicina  gi|29409260  .......... .......... .....T.... .......... .......... ....
Calliphora vicina  gi|29409258  .......... .......... .....T.... .......... .......... ....
Calliphora vicina  gi|29409256  .......... .......... .....T.... .......... .......... ....
Calliphora vicina  gi|29409254  .......... .......... .....T.... .......... .......... ....
Calliphora vomitoria gi|442388  .......... ...T...... .......... .......... ...T.....T ..A.
Calliphora vomitoria gi|294092  .......... ...T...... .......... .......... ...T.....T ..A.
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Calliphora vomitoria gi|294092  ......... ...T..... ......... ......... ...T.....T ..A.
Calliphora vomitoria gi|294092  ......... ...T..... ......... ......... ...T.....T ..A.
Calliphora vomitoria gi|294092  ......... ...T..... ......... ......... ...T.....T ..A.
Calliphora vomitoria gi|294092  ......... ...T..... ......... ......... ...T.....T ..A.
Calliphora vomitoria gi|294092  ......... ...T..... ......... ......... ...T.....T ..A.
Calliphora vomitoria gi|294092  ......... ...T..... ......... ......... ...T.....T ..A.
Eucalliphora latifrons gi|1132  ......... ...C.....T ........T. ......... ......T..T C.A.
Lucilia adisoemartoi gi|198795  ....C..... ...T.....T ......... ......... ...T.....T ..A.
Lucilia ampullacea gi|29409234  ....C..... ...T.....T ........T. ......... .........T C.A.
Lucilia ampullacea gi|29409232  ....C..... ...T.....T ........T. ......... .........T C.A.
Lucilia ampullacea gi|29409230  ....C..... ...T.....T ........T. ......... .........T C.A.
Lucilia ampullacea gi|29409228  ....C..... ...T.....T ........T. ......... .........T C.A.
Lucilia ampullacea gi|29409226  ....C..... ...T.....T ........T. ......... .........T C.A.
Lucilia bazini gi|33667901|     ....C..... ...T..... ......... ......... .........T ..A.
Lucilia caesar gi|29409218|     ......... ...T..... ......... ......... .........T ..A.
Lucilia caesar gi|29409216|     ......... ...T..... ......... ......... .........T ..A.
Lucilia caesar gi|21727823|     ......... ...T..... .....T.... ......... .........T ..A.
Lucilia caesar gi|29409224      ......... ...T..... ......... ......... .........T ..A.
Lucilia caesar gi|29409222|     ......... ...T..... ......... ......... .........T ..A.
Lucilia caesar gi|29409220      ......... ...T..... ......... ......... .........T ..A.
Lucilia cuprina gi|21727832|    ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|21727835|    ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|21727826|    ......... ......... .....T..T. ......... ...T.....T ..A.
Lucilia cuprina gi|4204934      ......... ......... .....T..T. ......... .........T ..A.
Lucilia cuprina gi|4204926|     ......... ......... .....T..T. ......... ...T.....T ..A.
Lucilia cuprina gi|4204932|     ......... ......... .....TG.CT ......... ...T.....T ..A.
Lucilia cuprina gi|4204940|     ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|4204936      ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|4204930|     ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|4204928|     ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|4204938      ......... ......... ......... ......... .........T .CA.
Lucilia cuprina gi|21727844|    ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|21727838|    ......... ......... ......... ......... ...T.....T ..A.
Lucilia cuprina gi|39652363     ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|39652343     ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|39652341     ......... ......... ......... ......... .........T ..A.
Lucilia cuprina gi|21386902|    ......... ......... .....T..T. .G..... ...T.....T ..A.
Lucilia cuprina gi|21727829|    ......... ......... .....T..T. ......... ...T.....T ..A.
Lucilia cuprina gi|21727841|    ......... ......... ......... ......... .........T ..A.
Lucilia hainanensis gi|3366790  .G..... ...T.....T .....T.... .C..... ...T.....T ..A.
Lucilia illustris gi|36788203   ......... ...T..... ......... ......... .........T ..A.
Lucilia illustris gi|29409250|  ......... ...T..... .....T.... ......... .........T ..A.
Lucilia illustris gi|29409244|  ......... ...T..... .....T.... ......... .........T ..A.
Lucilia illustris gi|29409236   ......... ...T..... .....T.... ......... .........T ..A.
Lucilia illustris gi|29409248|  ......... ...T..... .....T.... ......... .........T ..A.
Lucilia illustris gi|29409252|  ......... ...T..... .....T.... ......... .........T ..A.
Lucilia illustris gi|29409242|  ......... ...T..... .....T.... ......... .........T ..A.
Lucilia illustris gi|29409240   ......... ...T..... .....T.... ......... .........T ..A.
Lucilia illustris gi|29409238|  ......... ...T..... .....T.... ......... .........T ..A.
Lucilia illustris gi|29409246|  ......... ...T..... .....T.... ......... .........T ..A.
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Lucilia porphyrina gi|21386904  ....C..... ...T.....T ........T. .......... .........T ..A.
Lucilia porphyrina gi|19879590  ....C..... ...C.....T ........T. .......... .........T ..A.
Lucilia sericata gi|21727847    .......... .......... .....T..T. .......... ...T.....T ..A.
Lucilia sericata gi|21727859|   .......... .......... .....T..T. .......... ...T.....T ..A.
Lucilia sericata gi|21727850|   .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|21727853    .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|21727856|   .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|21727862|   .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|29409284    .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|29409282|   .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|29409286|   .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|29409290    .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|39652365    .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|39652355    .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|39652325    .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|39652323    .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|29409288|   .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|39652303    .......... .......... ........T. .......... ...T.....T ..A.
Lucilia sericata gi|39652337    .......... .......... .....T..T. .......... ...T.....T ..A.
Chrysomya albiceps gi|39652345  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|39652339  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|39652321  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|39652315  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|39652309  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|3659514|  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|39652367  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|39652353  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|39652317  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya albiceps gi|39652335  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya bezziana gi|11321181  .....T.A.. C..T...... .......... .......... ...T..T..T ..A.
Chrysomya inclinata gi|3965235  .......A.. ...T...... .......... .......... .........T ..A.
Chrysomya marginalis gi|396523  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya marginalis gi|396523  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya marginalis gi|396523  .....T.A.. ...T...... .....T.... .......G.. ...T..T..T ..A.
Chrysomya marginalis gi|396523  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya marginalis gi|396523  .....T.A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya marginalis gi|396523  .....T.A.. ...T...... .......... .......... ...T..T..T ..A.
Chrysomya megacephala gi|39652  .......A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya megacephala gi|39652  .......A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya megacephala gi|20453  .......A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya megacephala gi|39652  .......A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya megacephala gi|11321  .......A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya megacephala gi|39652  .......A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya megacephala gi|39652  .......A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya megacephala gi|39652  .......A.. ...T...... .....T.... .......... ...T..T..T ..A.
Chrysomya megacephala gi|39652  .......A.. ...T...... .......... .......... ...T..T..T ..A.
Chrysomya norrisi gi|11321193|  .....T.A.. .......... .....T.... .T........ ...T..T..T ..A.
Chrysomya pinguis gi|20453018|  .....T.A.. ...T...... ..C..... .......... ......T..T ..A.
Chrysomya putoria gi|39652347   .....T.A.. C..T...... .......... .......... ...T..T..T ..A.
Chrysomya putoria gi|39652307   .....T.A.. C..T...... .......... .......... ...T..T..T ..A.
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Chrysomya putoria gi|11321199|  .....T.A.. C..T..... ......... ......... ...T..T..T ..A.
Chrysomya putoria gi|39652357   .....T.A.. C..T..... ......... ......... ...T..T..T ..A.
Chrysomya putoria gi|39652299   .....T.A.. C..T..... ......... ......... ...T..T..T ..A.
Chrysomya rufifacies gi|365951  .....T.A.. ...T..... .....T.... .......G.. ......T..T ..A.
Chrysomya rufifacies gi|396523  .....T.A.. ...T..... .....T.... .......G.. ......T..T ..A.
Chrysomya rufifacies gi|396522  .....T.A.. ...T..... .....T.... .......G.. ......T..T ..A.
Chrysomya semimetallica gi|113  .......A.. ...T..... ..C..T..G. .......G.. .........T ..A.
Chrysomya varipes gi|39652375   .....T.A.. ...T..... .....T.... ......... T..T..T..T ..A.
Chrysomya varipes gi|39652373   .....T.A.. ...T..... ......... ......... T..T..T..T ..A.
Chrysomya varipes gi|39652371   .....T.A.. ...T..... .....T.... ......... T..T..T..T ..A.
Chrysomya varipes gi|11321205|  .....T.A.. ...T..... .....T.... ......... T.....T..T ..A.
Cochliomyia macellaria gi|1132  .....T.A.. C..T..... .....T.... .C....... ...T.....T ..A.
Phormia regina gi|8272632       .....T.A.. C..T..... ......... ......... ...T..T..T ..A.
Phormia regina gi|11321187|     .....T.A.. C..T..... ......... ......... ...T..T..T ..A.
Drosophila yakuba               .....T.A.. ...T..T..T .....T.... .T....... ...T..T..T ....
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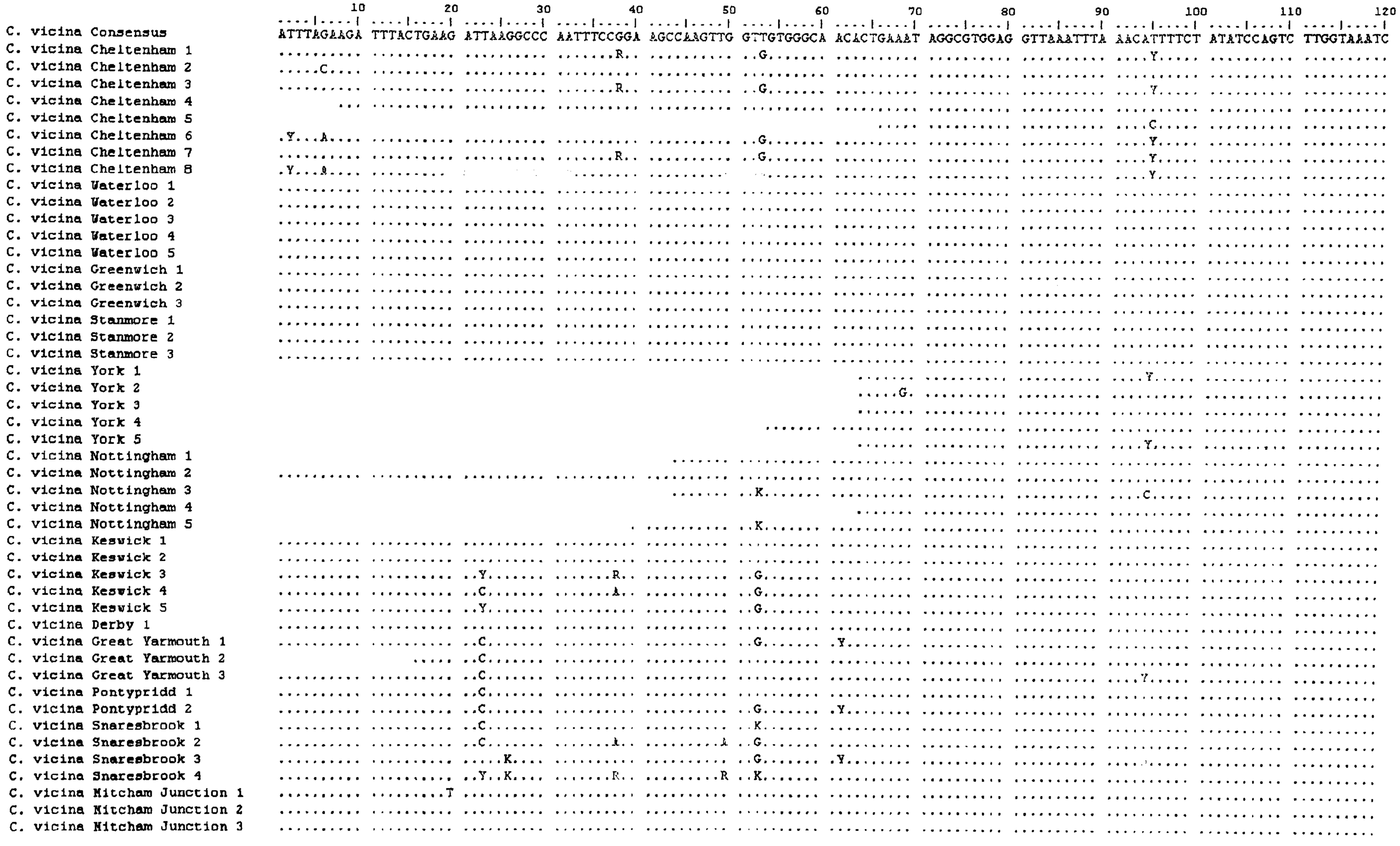
Appendix V.

Alignment of partial XDH gene for *C. vicina* and *C. vomitoria* samples. Dot indicates similar nucleotide to first sequence in alignment.



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C. vicina Mitcham Junction 4    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Mitcham Junction 5    .......... .......... ..C....... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Nonsuch Park 1        .......... .......... ..C....... .......R.. .........R ..G..... .......... .......... .......... .......... .......... ..........
C. vicina Nonsuch Park 2        .Y........ .........T NN...C.... .......... .......... ..K..... .......... .......... .......... .......... .......... ..........
C. vicina Nonsuch Park 3        .......... .........T .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Nonsuch Park 4        .......... .......... ..C....... .......R.. .........R ..G..... .......... .......... .......... .......... .......... ..........
C. vicina Nonsuch Park 5        .......... .........T .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 1           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 2           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 3           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 4           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 5           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 6           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 7           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 8           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 9           .....A.... .......... ..C....... .......A.. .........A ..G..... .......... .......... ..G..... ....C..... .......... ..........
C. vicina Wimbledon 10          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 11          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 12          .....A.... .......... ..C....... .......A.. .........A ..G..... .......... .......... ..G..... ....C..... .......... ..........
C. vicina Wimbledon 13          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vomitoria Consensus          .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria York 1             .C........ ......T... .CA.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Keswick 1                                                      .....A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Keswick 2                                                       ....A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Keswick 3                                                           G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Keswick 4                                                      .....A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Pett Wood 1         .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Pett Wood 2                                                         ..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Pett Wood 3         .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Pett Wood 4                                                    ...A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Pett Wood 5         .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Nonsuch Park 1                                                                 .... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Nonsuch Park 2      .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Nonsuch Park 3      .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Nonsuch Park 4      .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Nonsuch Park 5      .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Nonsuch Park 6      .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Nonsuch Park 7      .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 1           .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 2           .Y........ ......T... ..A.....A. .......A.. .........A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 3           .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 4           .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 5           .Y........ ......T... ..A.....A. .......A.. .........A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 6           .Y........ ......T... ..A.....A. .......A.. .........A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 7           .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 8           .C........ ......T... ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria Boxhill 9           .Y........ ......T... ..A.....A. .......A.. .........A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria 1                   .C........ ......T... ..A..K..A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria 2                   .C........ ......T..T ..A.....A. .......A.. ......A..A ..G..... .......... .......... .......... ....C..... .......... ..........
C. vomitoria 3                                                              ..A ..G..... .......... .......... .......... ....C..... .......... ..........
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C. vomitoria 4                  .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 5                                                                                  ..... ..... ..... ....C..... ..... .....
C. vomitoria 6                  .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 7                  .C..... .....T..T ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 8                                    .           .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 9                  .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 10                 .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 11                 .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 12                 .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 13                 .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria 14                 .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Mitcham Junction   .C..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Mitcham Junction   .Y..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Mitcham Junction   .Y..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Mitcham Junction   .C..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Mitcham Junction   .C..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Mitcham Junction   .C..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Mitcham Junction   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Hamstead Heath 1   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Hamstead Heath 2   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Hamstead Heath 3   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Hamstead Heath 4   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Hamstead Heath 5   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Hamstead Heath 6   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Wimbledon Common   .Y..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Wimbledon Common   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Wimbledon Common           .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Wimbledon Common   .Y..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Wimbledon Common   .Y..... .....T... ..A.....A. .....A.. .........A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Wimbledon Common   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Wimbledon Common   .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Cheltenham 1       .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Cheltenham 2       .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria East Sheen 1       .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria East Sheen 2       .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria East Sheen 3       .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Stanmore 1                       .A.....     .....A.. ..  ..A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Stanmore 2         .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Stanmore 3         .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Stanmore 4         .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....
C. vomitoria Stanmore 5         .C..... .....T... ..A.....A. .....A.. .....A..A ..G..... ..... ..... ..... ....C..... ..... .....

                                        130       140       150       160       170       180       190       200       210       220       230       240
                                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C. vicina Consensus             CCACAAAAGT TAAGGAAATG ATTGATGTCC AGGAACTCGA GGATAGTATT TATTTTGGTG CTTCCGTTAG TTTAATGGAT ATAGATAGGA TTTTAAGATC CAGCATAGAA AAGCTACCCG
C. vicina Cheltenham 1          .......... .......... .......... .....Y.... .......... .......... .......... .......... .....Y.... .......... .......... ..........
C. vicina Cheltenham 2          .......... .......... .......... .......... .......... .......... .......... .......... ........K. .......... .......... ..........
C. vicina Cheltenham 3          .......... .......... .......... .....T.... .......... .......... .......... .......... .....Y.... .......... .......... ..........
C. vicina Cheltenham 4          .......... .......... .......... .......... .......... .......... .......... .......... ........K. .......... .......... ..........
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C. vicina Cheltenham 5      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Cheltenham 6      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Cheltenham 7      ..........  ..........  ..........  .....Y....  ..........  ..........  ..........  ..........  .....Y....  ..........  ..........  ..........
C. vicina Cheltenham 8      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Waterloo 1        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Waterloo 2        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Waterloo 3        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Waterloo 4        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Waterloo 5        ..........  ..........  ..........  .....Y....  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Greenwich 1       ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Greenwich 2       ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Greenwich 3       ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Stanmore 1        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Stanmore 2        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Stanmore 3        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 1            ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 2            ..........  G.........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 3            ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 4            ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina York 5            ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Nottingham 1      ..........  ..........  ..........  .....Y....  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Nottingham 2      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Nottingham 3      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Nottingham 4      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Nottingham 5      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 1         ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 2         ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 3         ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .........K  ..........  ..........  ..........
C. vicina Keswick 4         ..........  ..........  ..........  .....T....  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Keswick 5         ..........  ..........  ..........  .....Y....  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Derby 1           ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Great Yarmouth 1  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Great Yarmouth 2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Great Yarmouth 3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Pontypridd 1      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Pontypridd 2      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Snaresbrook 1     ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Snaresbrook 2     ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Snaresbrook 3     ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Snaresbrook 4     ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Mitcham Junction 1 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Mitcham Junction 2 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ....W.....  ..........
C. vicina Mitcham Junction 3 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Mitcham Junction 4 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Mitcham Junction 5 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .........K  ..........  ..........  ..........
C. vicina Nonsuch Park 1    ..........  G.........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ....W.....  ..........
C. vicina Nonsuch Park 2    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ....W.....  ..........
C. vicina Nonsuch Park 3    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Nonsuch Park 4    ..........  G.........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C. vicina Nonsuch Park 5    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ....W.....  ..........
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C. vicina Wimbledon 1          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 2          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 3          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 4          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 5          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 6          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 7          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 8          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 9          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 10         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 11         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 12         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vicina Wimbledon 13         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C. vomitoria Consensus         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria York 1            .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Keswick 1         .......... A......... .......... .....T.... .........A .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Keswick 2         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Keswick 3         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Keswick 4         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Pett Wood 1       .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Pett Wood 2       .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Pett Wood 3       .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Pett Wood 4       .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Pett Wood 5       .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Nonsuch Park 1    .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Nonsuch Park 2    .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Nonsuch Park 3    .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Nonsuch Park 4    .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Nonsuch Park 5    .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Nonsuch Park 6    .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Nonsuch Park 7    .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 1         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 2         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 3         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 4         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 5         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 6         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 7         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 8         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria Boxhill 9         .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 1                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 2                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 3                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 4                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 5                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 6                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 7                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 8                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 9                 .......... A......... .......... .....T.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
C. vomitoria 10                .......... A......... .......... .....Y.... .......... .......... ....A..... .......... .....C.... .A........ .C.G..T... ..........
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C. vomitoria 11                  ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria 12                  ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria 13                  ..........A.........Y.....A.....C.....A.....C.G..T.....
C. vomitoria 14                  ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Mitcham Junction    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Mitcham Junction    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Mitcham Junction    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Mitcham Junction    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Mitcham Junction    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Mitcham Junction    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Mitcham Junction    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Hamstead Heath 1    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Hamstead Heath 2    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Hamstead Heath 3    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Hamstead Heath 4    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Hamstead Heath 5    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Hamstead Heath 6    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Wimbledon Common    ..........A.........T.....W.....A.....C.....A.....C.G..T.....
C. vomitoria Wimbledon Common    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Wimbledon Common    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Wimbledon Common    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Wimbledon Common    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Wimbledon Common    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Wimbledon Common    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Wimbledon Common    ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Cheltenham 1        ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Cheltenham 2        ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria East Sheen 1        ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria East Sheen 2        ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria East Sheen 3        ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Stanmore 1          ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Stanmore 2          ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Stanmore 3          ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Stanmore 4          ..........A.........T.....A.....C.....A.....C.G..T.....
C. vomitoria Stanmore 5          ..........A.........T.....A.....C.....A.....C.G..T.....

                                       250       260       270       280       290       300       310       320       330       340       350
                                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|
C. vicina Consensus              AACATCAAAC AAGATTTTTC CAATGTGCCG TCAATATGTT GCATTATTTT GCTGGTAAAC AAATTCGTAA TGTGGCGTCA TTGGGTGGCA ATATTATGAC GGGTAGTCCC ATATC
C. vicina Cheltenham 1           ..........S.........R.....S.....
C. vicina Cheltenham 2           ..........R.........S.....S.....
C. vicina Cheltenham 3           ..........S.........R.....S.....
C. vicina Cheltenham 4           ..........R.........S.....S.....
C. vicina Cheltenham 5           ..........S.........R.....S.....
C. vicina Cheltenham 6           ..........S.........R.....S.....
C. vicina Cheltenham 7           ..........S.........R.....S.....
C. vicina Cheltenham 8           ..........S.........R.....S.....
C. vicina Waterloo 1             ..........S.........R.....S.....
C. vicina Waterloo 2             ..........S.........R.....S.....
C. vicina Waterloo 3             ..........S.........R.....S.....
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C. vicina Waterloo 4              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Waterloo 5              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Greenwich 1             .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Greenwich 2             .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Greenwich 3             .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Stanmore 1              .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Stanmore 2              .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Stanmore 3              .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina York 1                  .......... .......... .......... .M........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina York 2                  .......... .......... .......... .......... .......... .......... .......... .......... .......... .Y........ .......... .....
C. vicina York 3                  .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina York 4                  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina York 5                  .......... .......... .........T. .......... .......... .......... .......... .......... .......... .......... .........A .....
C. vicina Nottingham 1            .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Nottingham 2            .......... .......... .......... .S........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Nottingham 3            .......... .......... .......... .S........ .......... .......... .......... .......... .......... .......... .........A .....
C. vicina Nottingham 4            .......... .......... .......... .A........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Nottingham 5            .......... .......... .......... .A........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Keswick 1               .......... .......... .......... .S........ .......... .......... .......... Y......... .......... .......... .......... .....
C. vicina Keswick 2               .......... .......... .......... .S........ .......... .......... .......... .......... .......... .......... .......... ...C.
C. vicina Keswick 3               .......... .......... .......... .A........ R......... .......... .......... .......... .......... .......... .......... .....
C. vicina Keswick 4               .......... .......... .......... .S........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Keswick 5               .......... .......... .......... .S........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Derby 1                 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Great Yarmouth 1        .......... .......... .......... .M........ R......... .......... .......... .......... .......... .......... .......... .....
C. vicina Great Yarmouth 2        .......... .......... .......... .A........ .......... .....S.... .......... .......... .......... .......... .........A ....
C. vicina Great Yarmouth 3        .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Pontypridd 1            .......... .......... .......... .M........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Pontypridd 2            .......... .......... .......... .M........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Snaresbrook 1           .......... .......... .......... .M........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Snaresbrook 2           .......... .......... .......... .A........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Snaresbrook 3           .......... .......... .......... .S........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Snaresbrook 4           .......... .......... .......... .M........ R......... .......... .......... .......... .......... .......... .......... .....
C. vicina Mitcham Junction 1      .......... .......... .......... .R........ .......... .......... .......... .......... .......... .......... .........A .....
C. vicina Mitcham Junction 2      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Mitcham Junction 3      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Mitcham Junction 4      .......... .......... .........T. .......... .......... .......... .......... .......... .......... .......... .........A .....
C. vicina Mitcham Junction 5      .......... .......... .......... .A........ R......... .......... .......... .......... .......... .......... .......... .....
C. vicina Nonsuch Park 1          .......... .......... .......... .......... .......... .......... .......... .......... .......... .Y........ .......... .....
C. vicina Nonsuch Park 2          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Nonsuch Park 3          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Nonsuch Park 4          .......... .......... .......... .......... .......... .......... .......... .......... .......... .Y........ .......... .....
C. vicina Nonsuch Park 5          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Wimbledon 1             .......... .......... .......... .G........
C. vicina Wimbledon 2             .......... .......... .......... .G........ .......... .......... ........
C. vicina Wimbledon 3             .......... .......... .......... .G........ .......... .
C. vicina Wimbledon 4             .......... .......... .......... .G........ .......... ........
C. vicina Wimbledon 5             .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Wimbledon 6             .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
C. vicina Wimbledon 7             .......... .......... .......... .G........ .......... .......... .......... .......... .......... .......... .......... .....
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C. vicina Wimbledon 8          ..........  ..........  ..........  .G........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....
C. vicina Wimbledon 9          ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....
C. vicina Wimbledon 10         ..........  ..........  ..........  .G........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....
C. vicina Wimbledon 11         ..........  ..........  ..........  .G........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....
C. vicina Wimbledon 12         ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....
C. vicina Wimbledon 13         ..........  ..........  .........T.  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....
C. vomitoria Consensus         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria York 1            ..........  ..........  .........A  .. ..... .  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Keswick 1         ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  ...C.
C. vomitoria Keswick 2         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  ...C.
C. vomitoria Keswick 3         ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Keswick 4         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Pett Wood 1       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Pett Wood 2       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Pett Wood 3       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Pett Wood 4       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Pett Wood 5       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Nonsuch Park 1    ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Nonsuch Park 2    ..........  ..........  .........A  ..........  ...C......
C. vomitoria Nonsuch Park 3    ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Nonsuch Park 4    ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Nonsuch Park 5    ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Nonsuch Park 6    ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Nonsuch Park 7    ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 1         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 2         ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 3         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 4         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 5         ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 6         ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 7         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 8         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Boxhill 9         ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 1                 ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 2                 ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 3                 ..........  ..........  .........A  ..........  ...C.....Y  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 4                 ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 5                 ..........  ..........  .........A  ..........  ...C.....C  ..........  .M........  ..........  C.........  .C........  ..........  .....
C. vomitoria 6                 ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 7                 ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 8                 ..........  ..........  .........A  ..........  ...C.....Y  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 9                 ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 10                ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 11                ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 12                ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 13                ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria 14                ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Mitcham Junction  ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Mitcham Junction  ..........  ..........  .........A  ..........  ...C.....Y  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Mitcham Junction  ..........  ..........  .........A  ..........  ...C.....Y  ..........  ..........  ..........  C.........  .C........  ..........  .....
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C. vomitoria Mitcham Junction   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Mitcham Junction   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Mitcham Junction   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Mitcham Junction   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Hamstead Heath 1   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Hamstead Heath 2   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Hamstead Heath 3   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Hamstead Heath 4   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Hamstead Heath 5   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Hamstead Heath 6   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Wimbledon Common   ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Wimbledon Common   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Wimbledon Common   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Wimbledon Common   ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Wimbledon Common   ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Wimbledon Common   ..........  ..........  .........A  ..........  ...C......  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Wimbledon Common   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Wimbledon Common   ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Cheltenham 1       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Cheltenham 2       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria East Sheen 1       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria East Sheen 2       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria East Sheen 3       ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Stanmore 1         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Stanmore 2         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Stanmore 3         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Stanmore 4         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
C. vomitoria Stanmore 5         ..........  ..........  .........A  ..........  ...C.....C  ..........  ..........  ..........  C.........  .C........  ..........  .....
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Allele frequencies at the different XDH loci for each of the *C. vicina* populations.

Nucleotide Position	Nucleotide	Cheltenham	Waterloo	Greenwich	Stanmore	York	Nottingham	Keswick	Derby	G Yarmouth	Pontypridd	Snaresbrook	Mitcham J	Nonsuch Pk	Wimbledon
690	T	0.875	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.900	1.000
	C	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000
694	G	0.625	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.846
	C	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	A	0.250	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.154
708	G	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.800	0.400	1.000
	T	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.600	0.000
711	T	1.000	1.000	1.000	1.000	1.000	1.000	0.600	1.000	0.000	0.000	0.375	0.800	0.600	0.846
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.400	0.000	1.000	1.000	0.625	0.200	0.400	0.154
714	G	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.750	1.000	0.800	1.000
	T	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	0.000	0.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.000
726	G	0.813	1.000	1.000	1.000	1.000	1.000	0.700	1.000	1.000	1.000	0.625	1.000	0.800	0.846
	A	0.188	0.000	0.000	0.000	0.000	0.000	0.300	0.000	0.000	0.000	0.375	0.000	0.200	0.154
738	T	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.625	1.000	0.800	0.846
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.375	0.000	0.200	0.154
741	T	0.500	1.000	1.000	1.000	1.000	0.800	0.400	1.000	0.667	0.500	0.250	1.000	0.500	0.846
	G	0.500	0.000	0.000	0.000	0.000	0.200	0.600	0.000	0.333	0.500	0.750	0.000	0.500	0.154
750	C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.833	0.750	0.875	1.000	1.000	1.000
	T	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.250	0.125	0.000	0.000	0.000
757	A	1.000	1.000	1.000	1.000	0.800	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	G	0.000	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
771	T	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.846
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.154
783	T	0.563	1.000	1.000	1.000	0.800	0.800	1.000	1.000	0.833	1.000	1.000	1.000	1.000	0.846
	C	0.438	0.000	0.000	0.000	0.200	0.200	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.154
819	T	1.000	1.000	1.000	1.000	0.800	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.600	1.000
	G	0.000	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.400	0.000
844	C	0.750	1.000	1.000	1.000	1.000	0.900	0.700	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	T	0.250	0.000	0.000	0.000	0.000	0.100	0.300	0.000	0.000	0.000	0.000	0.000	0.000	0.000
894	T	0.813	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	C	0.188	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
897	G	0.875	1.000	1.000	1.000	1.000	1.000	0.900	1.000	1.000	1.000	1.000	0.900	1.000	1.000
	T	0.125	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.100	0.000	0.000
913	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.900	0.700	1.000
	T	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.300	0.000
957	C	1.000	1.000	1.000	1.000	0.800	1.000	1.000	1.000	1.000	1.000	1.000	0.800	1.000	0.923
	T	0.000	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.000	0.077
960	C	0.563	1.000	0.000	0.000	0.700	0.200	0.400	1.000	0.500	0.500	0.375	0.600	1.000	0.231
	A	0.313	0.000	1.000	1.000	0.200	0.400	0.400	0.000	0.000	0.000	0.125	0.100	0.000	0.769
	G	0.125	0.000	0.000	0.000	0.100	0.400	0.200	0.000	0.500	0.500	0.500	0.300	0.000	0.000
969	G	1.000	1.000	1.000	1.000	1.000	1.000	0.900	1.000	0.833	1.000	0.875	0.900	1.000	1.000
	A	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.167	0.000	0.125	0.100	0.000	0.000
984	T	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.667	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000
999	T	1.000	1.000	1.000	1.000	1.000	1.000	0.900	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1020	T	1.000	1.000	1.000	1.000	0.900	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.800	1.000
	C	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.000
1038	C	1.000	1.000	1.000	1.000	0.800	0.800	1.000	1.000	0.667	1.000	1.000	0.600	1.000	1.000
	A	0.000	0.000	0.000	0.000	0.200	0.200	0.000	0.000	0.333	0.000	0.000	0.400	0.000	0.000
1042	T	1.000	1.000	1.000	1.000	1.000	1.000	0.800	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Allele frequencies at the different XDH loci for each of the *C. vomitoria* populations.

Nucleotide Position	Nucleotide	York	Keswick	Petts Wood	Nonsuch Park	Box Hill	Commercial	Mitcham Junction	Hampstead Heath	Wimbledon Common	Cheltenham	East Sheen	Stanmore
690	C	1.000	1.000	1.000	1.000	0.778	1.000	0.857	1.000	0.750	1.000	1.000	1.000
	T	0.000	0.000	0.000	0.000	0.222	0.000	0.143	0.000	0.250	0.000	0.000	0.000
708	G	1.000	1.000	1.000	1.000	1.000	0.857	1.000	1.000	1.000	1.000	1.000	1.000
	T	0.000	0.000	0.000	0.000	0.000	0.143	0.000	0.000	0.000	0.000	0.000	0.000
710	T	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	C	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
714	G	1.000	1.000	1.000	1.000	1.000	0.964	1.000	1.000	1.000	1.000	1.000	1.000
	T	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000
735	A	1.000	1.000	1.000	1.000	0.556	0.929	0.000	1.000	0.500	1.000	1.000	1.000
	G	0.000	0.000	0.000	0.000	0.444	0.071	1.000	0.000	0.500	0.000	0.000	0.000
844	T	1.000	1.000	1.000	1.000	1.000	0.857	1.000	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.000	0.000	0.000	0.000	0.143	0.000	0.000	0.000	0.000	0.000	0.000
858	T	1.000	0.750	1.000	1.000	1.000	1.000	1.000	1.000	0.938	1.000	1.000	1.000
	A	0.000	0.250	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.000
978	C	1.000	0.500	1.000	1.000	0.556	0.929	0.857	1.000	0.500	1.000	1.000	1.000
	T	0.000	0.500	0.000	0.000	0.444	0.071	0.143	0.000	0.500	0.000	0.000	0.000
991	A	1.000	1.000	1.000	1.000	1.000	0.964	1.000	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000
1042	T	1.000	0.500	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.500	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Comparison of *C. vicina* populations. Tamura-Nei genetic distances between *C. vicina* populations below the diagonal (bottom left). Tamura-Nei genetic distances within populations on the diagonal. Geographic distances (km) between population trap locations above the diagonal (above right). N/a – not applicable. N/c – not calculated.

	Consensus	Cheltenham	Waterloo	Greenwich	Stanmore	York	Nottingham	Keswick	Derby	Great Yarmouth	Pontypridd	Snaresbrook	Mitcham Junction	Nonsuch Park	Wimbledon
Consensus	**n/c**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Cheltenham	0.0033	**0.0028**	142	150	125	241	210	335	139	272	92	149	143	140	136
Waterloo	0.0000	0.0032	**0.0000**	8	19	280	174	399	193	176	223	12	13	18	13
Greenwich	0.0028	0.0045	0.0028	**0.0000**	27	286	181	406	200	174	231	12	14	20	18
Stanmore	0.0028	0.0045	0.0028	0.0000	**0.0000**	265	157	381	176	177	209	23	27	29	21
York	0.0035	0.0037	0.0035	0.0048	0.0048	**0.0066**	114	152	102	240	304	275	292	292	286
Nottingham	0.0033	0.0031	0.0033	0.0026	0.0026	0.0051	**0.0033**	226	20	199	210	171	184	186	178
Keswick	0.0046	0.0047	0.0045	0.0046	0.0046	0.0047	0.0034	**0.0052**	207	391	335	397	408	409	402
Derby	0.0000	0.0033	0.0000	0.0028	0.0028	0.0035	0.0033	0.0046	**n/c**	216	210	190	203	204	197
Great Yarmouth	0.0058	0.0062	0.0058	0.0068	0.0068	0.0046	0.0036	0.0057	0.0058	**0.0049**	365	164	187	193	184
Pontypridd	0.0043	0.0051	0.0043	0.0043	0.0043	0.0028	0.0025	0.0043	0.0043	0.0024	**0.0028**	232	221	216	214
Snaresbrook	0.0050	0.0044	0.0050	0.0050	0.0050	0.0035	0.0030	0.0039	0.0050	0.0044	0.0029	**0.0029**	24	30	25
Mitcham Junction	0.0034	0.0062	0.0034	0.0051	0.0051	0.0050	0.0043	0.0068	0.0034	0.0066	0.0060	0.0069	**0.0060**	6	8
Nonsuch Park	0.0057	0.0066	0.0057	0.0086	0.0086	0.0043	0.0058	0.0075	0.0057	0.0084	0.0063	0.0065	0.0080	**0.0075**	8
Wimbledon	0.0061	0.0065	0.0061	0.0044	0.0044	0.0058	0.0048	0.0071	0.0061	0.0087	0.0070	0.0071	0.0079	0.0112	**0.0084**

Comparison of *C. vomitoria* populations. Tamura-Nei genetic distances between *C. vomitoria* populations below the diagonal (bottom left). Tamura-Nei genetic distances within populations on the diagonal. Geographic distances (km) between population trap locations above the diagonal (above right). N/a – not applicable. N/c – not calculated.

	Consensus	York	Keswick	Petts Wood	Nonsuch Park	Box Hill	Commercial Supplier	Mitcham Junction	Hampstead Heath	Wimbledon Common	Cheltenham	East Sheen	Stanmore
Consensus	**n/c**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
York	0.0029	**n/c**	152	297	294	305	n/a	292	274	288	241	283	265
Keswick	0.0041	0.0041	**0.0060**	417	409	418	n/a	408	392	405	312	398	381
Petts Wood	0.0000	0.0017	0.0041	**0.0000**	21	30	n/a	16	26	19	155	26	37
Nonsuch Park	0.0000	0.0025	0.0037	0.0000	**0.0000**	14	n/a	6	22	6	137	11	29
Box Hill	0.0025	0.0054	0.0051	0.0024	0.0022	**0.0032**	n/a	19	36	20	138	23	41
Commercial Supplier	0.0009	0.0031	0.0043	0.0007	0.0008	0.0029	**0.0013**	n/a	n/a	n/a	n/a	n/a	n/a
Mitcham Junction	0.0028	0.0057	0.0060	0.0023	0.0025	0.0025	0.0029	**0.0000**	18	4	140	11	27
Hampstead Heath	0.0000	0.0029	0.0041	0.0000	0.0000	0.0025	0.0009	0.0028	**0.0000**	16	137	14	12
Wimbledon Common	0.0028	0.0057	0.0051	0.0027	0.0025	0.0029	0.0031	0.0024	0.0028	**0.0033**	136	7	24
Cheltenham	0.0000	0.0029	0.0041	0.0000	0.0000	0.0025	0.0009	0.0028	0.0000	0.0028	**0.0000**	130	123
East Sheen	0.0000	0.0029	0.0041	0.0000	0.0000	0.0025	0.0009	0.0028	0.0000	0.0028	0.0000	**0.0000**	18
Stanmore	0.0000	0.0029	0.0041	0.0000	0.0000	0.0026	0.0008	0.0029	0.0000	0.0029	0.0000	0.0000	**0.0000**

Log likelihood values for *C. vicina* samples for each population. Black line outlines the assigned population. Bold font highlights match between assigned population and actual sample population.

Sample	Pop	Cheltenham	Waterloo	Greenwich	Stanmore	York	Nottingham	Keswick	Derby	GYarmouth	Pontypridd	Snaresbrook	MJunction	Nonsuch Park	Wimbledon
1	Cheltenham	**-3.462**	-12.495	-12.495	-12.495	-11.011	-7.025	-6.002	-12.495	-13.629	-13.648	-8.446	-13.016	-10.559	-7.292
2	Cheltenham	**-5.402**	-9.398	-7.398	-7.398	-8.158	-6.867	-7.777	-9.398	-13.231	-12.551	-9.956	-7.175	-12.860	-8.452
3	Cheltenham	**-4.241**	-14.796	-14.796	-14.796	-13.312	-8.280	-6.671	-14.796	-15.930	-15.949	-10.747	-15.317	-12.860	-9.593
4	Cheltenham	-4.004	-5.398	-3.398	-3.398	-4.158	-2.867	-3.777	-5.398	-9.231	-8.551	-5.956	-3.175	-8.860	-4.597
5	Cheltenham	-3.071	-4.000	-8.000	-8.000	-2.575	-3.275	-7.123	-4.000	-7.532	-9.454	-8.206	-5.743	-7.462	-3.840
6	Cheltenham	**-4.000**	-11.398	-15.398	-15.398	-11.370	-9.275	-10.169	-11.398	-12.833	-12.852	-10.650	-13.141	-9.814	-7.459
7	Cheltenham	**-3.462**	-12.495	-12.495	-12.495	-11.011	-7.025	-6.002	-12.495	-13.629	-13.648	-8.446	-13.016	-10.559	-7.292
8	Cheltenham	**-4.000**	-7.398	-11.398	-11.398	-7.370	-8.071	-10.521	-7.398	-12.231	-12.852	-11.604	-9.141	-9.814	-5.978
9	Waterloo	-2.852	**0.000**	-4.000	-4.000	-1.370	-2.071	-3.123	0.000	-6.134	-5.454	-4.206	-1.743	-3.462	-2.359
10	Waterloo	-2.852	**0.000**	-4.000	-4.000	-1.370	-2.071	-3.123	0.000	-6.134	-5.454	-4.206	-1.743	-3.462	-2.359
11	Waterloo	-2.852	**0.000**	-4.000	-4.000	-1.370	-2.071	-3.123	0.000	-6.134	-5.454	-4.206	-1.743	-3.462	-2.359
12	Waterloo	-2.852	**0.000**	-4.000	-4.000	-1.370	-2.071	-3.123	0.000	-6.134	-5.454	-4.206	-1.743	-3.462	-2.359
13	Waterloo	-2.852	**0.000**	-4.000	-4.000	-1.370	-2.071	-3.123	0.000	-6.134	-5.454	-4.206	-1.743	-3.462	-2.359
14	Greenwich	-3.363	-4.000	**0.000**	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
15	Greenwich	-3.363	-4.000	**0.000**	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
16	Greenwich	-3.363	-4.000	**0.000**	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
17	Stanmore	-3.363	-4.000	0.000	**0.000**	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
18	Stanmore	-3.363	-4.000	0.000	**0.000**	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
19	Stanmore	-3.363	-4.000	0.000	**0.000**	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
20	York	-3.013	-3.398	-5.398	-5.398	-2.216	-1.770	-4.822	-3.398	-6.231	-6.852	-5.479	-3.442	-6.860	-3.860
21	York	-12.551	-9.699	-13.699	-13.699	**-4.432**	-11.770	-12.822	-9.699	-15.833	-15.153	-13.905	-11.442	-8.115	-12.058
22	York	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
23	York	-2.852	0.000	-4.000	-4.000	-1.370	-2.071	-3.123	0.000	-6.134	-5.454	-4.206	-1.743	-3.462	-2.359
24	York	-10.660	-9.699	-13.699	-13.699	**-4.080**	-7.576	-12.822	-9.699	-11.134	-15.153	-13.905	-4.999	-13.161	-8.957
25	Nottingham	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
26	Nottingham	-2.807	-1.699	-1.699	-1.699	-1.613	**-1.469**	-2.822	-1.699	-7.532	-6.852	-4.382	-2.221	-5.161	-1.535
27	Nottingham	-6.724	-11.398	-11.398	-11.398	-5.721	**-4.178**	-10.345	-11.398	-9.532	-14.551	-11.604	-8.272	-12.860	-7.455
28	Nottingham	-4.159	-4.000	-4.000	-4.000	-3.061	**-1.469**	-3.725	-4.000	-6.134	-5.454	-3.956	-2.345	-7.462	-5.085
29	Nottingham	-4.034	-7.398	-7.398	-7.398	-6.459	**-2.423**	-3.315	-7.398	-7.833	-6.852	-4.877	-5.743	-8.860	-7.224
30	Keswick	-4.506	-3.398	-3.398	-3.398	-3.312	-3.168	-3.476	-3.398	-9.231	-8.551	-6.081	-3.919	-6.860	-3.234
31	Keswick	-6.807	-5.699	-5.699	-5.699	-5.613	-5.469	**-4.027**	-5.699	-11.532	-10.852	-8.382	-6.221	-9.161	-5.535
32	Keswick	-8.437	-14.796	-14.796	-14.796	-13.856	-9.469	**-4.622**	-14.796	-8.231	-8.250	-4.643	-9.652	-11.036	-10.843
33	Keswick	-9.034	-17.699	-17.699	-17.699	-17.613	-12.581	**-4.294**	-17.699	-12.134	-10.852	-7.428	-15.425	-10.717	-9.977
34	Keswick	-4.682	-9.097	-9.097	-9.097	-9.011	-5.025	**-2.412**	-9.097	-7.532	-6.250	-4.604	-8.221	-6.735	-5.154
35	Derby	-2.852	0.000	-4.000	-4.000	-1.370	-2.071	-3.123	**0.000**	-6.134	-5.454	-4.206	-1.743	-3.462	-2.359
36	Great Yarmouth	-10.250	-11.398	-15.398	-15.398	-12.768	-10.673	-5.476	-11.398	-3.532	-3.329	-3.897	-9.300	-7.212	-8.718
37	Great Yarmouth	-14.904	-13.398	-15.398	-15.398	-10.818	-10.372	-11.175	-13.398	**-3.338**	-8.852	-11.036	-6.999	-13.212	-12.601
38	Great Yarmouth	-7.967	-9.699	-9.699	-9.699	-7.362	-5.770	-5.777	-9.699	**-2.532**	-3.153	-5.212	-5.249	-9.513	-7.005
39	Pontypridd	-7.205	-5.699	-7.699	-7.699	-5.914	-5.469	-3.476	-5.699	-1.833	**-1.153**	-3.337	-2.948	-5.513	-4.902
40	Pontypridd	-8.904	-11.398	-13.398	-13.398	-11.613	-8.372	-4.822	-11.398	-2.833	**-1.329**	-2.926	-8.646	-7.212	-8.081
41	Snaresbrook	-6.904	-7.398	-9.398	-9.398	-7.613	-5.770	-2.998	-7.398	-1.833	-0.852	-2.559	-4.646	-5.212	-5.341
42	Snaresbrook	-13.432	-20.000	-20.000	-20.000	-19.061	-14.673	-8.461	-20.000	-10.736	-9.454	**-3.446**	-15.550	-10.222	-11.008
43	Snaresbrook	-6.205	-9.097	-9.097	-9.097	-9.011	-6.071	-5.868	-9.097	-10.231	-8.727	**-4.148**	-9.618	-8.462	-6.414
44	Snaresbrook	-10.035	-11.893	-13.893	-13.893	-12.108	-10.265	-6.639	-11.893	-9.026	-9.347	**-3.041**	-9.494	-8.638	-8.576
45	Mitcham Junction	-11.460	-11.699	-9.699	-9.699	-7.663	-6.372	-11.123	-11.699	-12.134	-14.852	-12.257	**-4.078**	-10.809	-10.898
46	Mitcham Junction	-4.551	-1.699	-5.699	-5.699	-3.069	-3.770	-4.822	-1.699	-7.833	-7.153	-5.905	-2.397	-3.529	-4.058
47	Mitcham Junction	-2.852	0.000	-4.000	-4.000	-1.370	-2.071	-3.123	0.000	-6.134	-5.454	-4.206	-1.743	-3.462	-2.359
48	Mitcham Junction	-10.852	-8.000	-12.000	-12.000	-3.779	-7.275	-11.123	-8.000	-10.736	-13.454	-12.206	**-3.300**	-11.462	-8.517
49	Mitcham Junction	-10.402	-11.398	-11.398	-11.398	-10.459	-8.867	-5.384	-11.398	-4.231	-4.852	-5.756	-4.856	-11.212	-9.964
50	Nonsuch Park	-14.586	-17.097	-21.097	-21.097	-14.626	-16.372	-10.588	-17.097	-11.833	-10.551	-8.349	-16.044	**-5.069**	-11.898
51	Nonsuch Park	-12.794	-13.097	-17.097	-17.097	-14.467	-13.770	-14.044	-13.097	-17.532	-16.551	-14.576	-10.999	**-4.733**	-14.196
52	Nonsuch Park	-8.551	-5.699	-9.699	-9.699	-7.069	-7.770	-8.822	-5.699	-11.833	-11.153	-9.905	-3.601	**-3.177**	-8.058
53	Nonsuch Park	-14.586	-17.097	-21.097	-21.097	-14.626	-16.372	-10.588	-17.097	-11.833	-10.551	-8.349	-16.044	**-5.069**	-11.898
54	Nonsuch Park	-8.551	-5.699	-9.699	-9.699	-7.069	-7.770	-8.822	-5.699	-11.833	-11.153	-9.905	-3.601	**-3.177**	-8.058
55	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
56	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
57	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
58	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
59	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
60	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
61	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
62	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
63	Wimbledon	-17.140	-28.000	-32.000	-32.000	-26.575	-24.479	-19.859	-28.000	-20.134	-21.454	-15.696	-26.948	-18.222	**-12.724**
64	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
65	Wimbledon	-3.363	-4.000	0.000	0.000	-2.459	-1.469	-3.123	-4.000	-9.532	-8.852	-5.161	-3.300	-7.462	-1.313
66	Wimbledon	-17.140	-28.000	-32.000	-32.000	-26.575	-24.479	-19.859	-28.000	-20.134	-21.454	-15.696	-26.948	-18.222	**-12.724**
67	Wimbledon	-6.852	-4.000	-8.000	-8.000	-2.575	-6.071	-7.123	-4.000	-10.134	-9.454	-8.206	-2.948	-7.462	-4.517

Log likelihood values for *C. vomitoria* samples for each population. Black line outlines the assigned population. Bold font highlights match between assigned population and actual sample population.

Sample	Population	York	Keswick	Petts Wood	Nonsuch Park	Box Hill	Commercial	M Junction	H Heath	W Common	Cheltenham	East Sheen	Stanmore
1	York	**0.000**	-5.454	-4.000	-4.000	-5.239	-4.326	-8.268	-4.000	-5.510	-4.000	-4.000	-4.000
2	Keswick	-16.000	**-2.408**	-12.000	-12.000	-9.433	-10.554	-13.824	-12.000	-7.862	-12.000	-12.000	-12.000
3	Keswick	-8.000	**-1.454**	-4.000	-4.000	-5.239	-4.326	-8.268	-4.000	-5.510	-4.000	-4.000	-4.000
4	Keswick	-8.000	-1.454	-4.000	-4.000	-1.433	-2.554	-5.824	-4.000	-1.510	-4.000	-4.000	-4.000
5	Keswick	-4.000	-1.454	**0.000**	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
6	Petts Wood	-4.000	-1.454	**0.000**	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
7	Petts Wood	-4.000	-1.454	**0.000**	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
8	Petts Wood	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
9	Petts Wood	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
10	Petts Wood	-4.000	-1.454	**0.000**	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
11	Nonsuch Park	-4.000	-1.454	0.000	**0.000**	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
12	Nonsuch Park	-4.000	-1.454	0.000	**0.000**	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
13	Nonsuch Park	-4.000	-1.454	0.000	**0.000**	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
14	Nonsuch Park	-4.000	-1.454	0.000	**0.000**	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
15	Nonsuch Park	-4.000	-1.454	0.000	**0.000**	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
16	Nonsuch Park	-4.000	-1.454	0.000	**0.000**	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	**0.000**
17	Nonsuch Park	-4.000	-1.454	0.000	**0.000**	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
18	Box Hill	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
19	Box Hill	-13.699	-7.153	-9.699	-9.699	-1.870	-6.481	-2.301	-9.699	-1.686	-9.699	-9.699	-9.699
20	Box Hill	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
21	Box Hill	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
22	Box Hill	-13.699	-7.153	-9.699	-9.699	-1.870	-6.481	-2.301	-9.699	-1.686	-9.699	-9.699	-9.699
23	Box Hill	-13.699	-7.153	-9.699	-9.699	-1.870	-6.481	-2.301	-9.699	-1.686	-9.699	-9.699	-9.699
24	Box Hill	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
25	Box Hill	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
26	Box Hill	-13.699	-7.153	-9.699	-9.699	-1.870	-6.481	-2.301	-9.699	-1.686	-9.699	-9.699	-9.699
27	Commercial	-5.699	-3.153	-1.699	-1.699	-2.938	**-1.456**	-5.967	-1.699	-3.209	-1.699	-1.699	-1.699
28	Commercial	-8.000	-5.454	-4.000	-4.000	-5.239	**-1.882**	-8.268	-4.000	-5.510	-4.000	-4.000	-4.000
29	Commercial	-5.699	-1.153	-1.699	-1.699	-1.035	-1.139	-4.745	-1.699	-1.209	-1.699	-1.699	-1.699
30	Commercial	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
31	Commercial	-5.699	-3.153	-1.699	-1.699	-2.938	**-1.456**	-5.967	-1.699	-3.209	-1.699	-1.699	-1.699
32	Commercial	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
33	Commercial	-8.000	-5.454	-4.000	-4.000	-5.239	**-1.882**	-8.268	-4.000	-5.510	-4.000	-4.000	-4.000
34	Commercial	-9.699	-5.153	-5.699	-5.699	-1.229	-3.367	-0.745	-5.699	-1.209	-5.699	-5.699	-5.699
35	Commercial	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
36	Commercial	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
37	Commercial	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
38	Commercial	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
39	Commercial	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
40	Commercial	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
41	Mitcham Junction	-8.000	-5.454	-4.000	-4.000	-1.433	-2.554	**-0.268**	-4.000	-1.510	-4.000	-4.000	-4.000
42	Mitcham Junction	-11.398	-6.852	-7.398	-7.398	-1.472	-5.066	**-1.222**	-7.398	-1.385	-7.398	-7.398	-7.398
43	Mitcham Junction	-11.398	-6.852	-7.398	-7.398	-1.472	-5.066	**-1.222**	-7.398	-1.385	-7.398	-7.398	-7.398
44	Mitcham Junction	-8.000	-5.454	-4.000	-4.000	-1.433	-2.554	**-0.268**	-4.000	-1.510	-4.000	-4.000	-4.000
45	Mitcham Junction	-8.000	-5.454	-4.000	-4.000	-1.433	-2.554	**-0.268**	-4.000	-1.510	-4.000	-4.000	-4.000
46	Mitcham Junction	-8.000	-5.454	-4.000	-4.000	-1.433	-2.554	**-0.268**	-4.000	-1.510	-4.000	-4.000	-4.000
47	Mitcham Junction	-8.000	-5.454	-4.000	-4.000	-1.433	-2.554	**-0.268**	-4.000	-1.510	-4.000	-4.000	-4.000
48	Hampstead Heath	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	**0.000**	-1.510	0.000	0.000	0.000
49	Hampstead Heath	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	**0.000**	-1.510	0.000	0.000	0.000
50	Hampstead Heath	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	**0.000**	-1.510	0.000	0.000	0.000
51	Hampstead Heath	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	**0.000**	-1.510	0.000	0.000	0.000
52	Hampstead Heath	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	**0.000**	-1.510	0.000	0.000	0.000
53	Hampstead Heath	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	**0.000**	-1.510	0.000	0.000	0.000
54	Wimbledon Common	-15.398	-7.329	-11.398	-11.398	-3.569	-8.180	-4.000	-11.398	**-2.561**	-11.398	-11.398	-11.398
55	Wimbledon Common	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
56	Wimbledon Common	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
57	Wimbledon Common	-13.699	-7.153	-9.699	-9.699	-1.870	-6.481	-2.301	-9.699	**-1.686**	-9.699	-9.699	-9.699
58	Wimbledon Common	-13.699	-7.153	-9.699	-9.699	-1.870	-6.481	-2.301	-9.699	**-1.686**	-9.699	-9.699	-9.699
59	Wimbledon Common	-13.699	-7.153	-9.699	-9.699	-1.870	-6.481	-2.301	-9.699	-1.686	-9.699	-9.699	-9.699
60	Wimbledon Common	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
61	Wimbledon Common	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	0.000
62	Cheltenham	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	**0.000**	0.000	0.000
63	Cheltenham	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	**0.000**	0.000	0.000
64	East Sheen	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	**0.000**	0.000
65	East Sheen	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	**0.000**	0.000
66	East Sheen	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	**0.000**	0.000
67	Stanmore	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	**0.000**
68	Stanmore	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	**0.000**
69	Stanmore	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	**0.000**
70	Stanmore	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	**0.000**
71	Stanmore	-4.000	-1.454	0.000	0.000	-1.239	-0.326	-4.268	0.000	-1.510	0.000	0.000	**0.000**

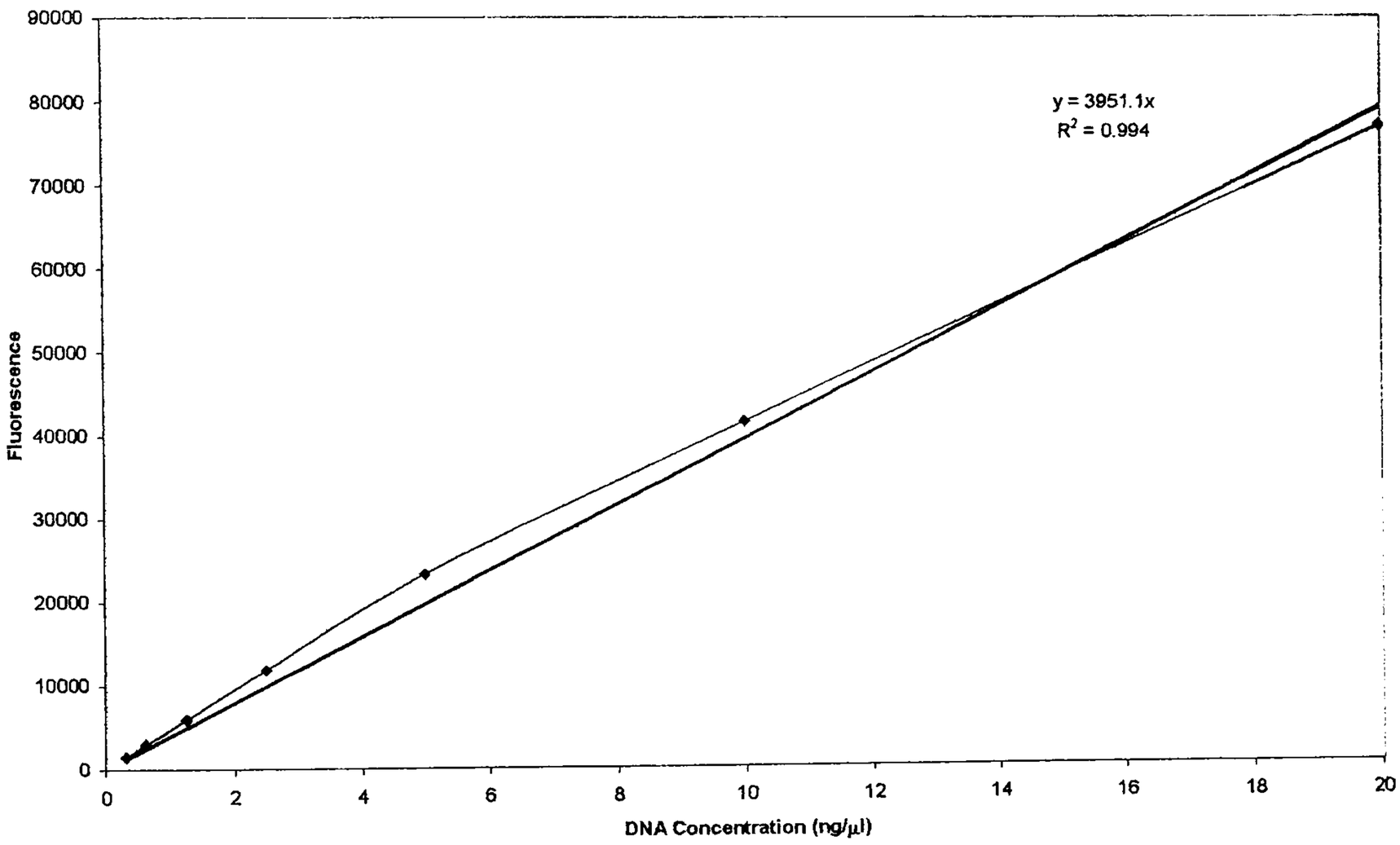
Appendix VI.

PicoGreen Fluorescence readings and corresponding standard calibration curves.

A.

	Sample	Reading 1	Minus Blank	Reading 2	Minus Blank	Average	Concentration (ng/ml)	Mean Conc.
	Standard	77213	76895.5	76989	76671.5	76783.5	20	
	Standard	43755	43437.5	39990	39672.5	41555	10	
	Standard	23906	23588.5	23309	22991.5	23290	5	
	Standard	12347	12029.5	12039	11721.5	11875.5	2.5	
	Standard	6413	6095.5	6131	5813.5	5954.5	1.25	
	Standard	3378	3060.5	3140	2822.5	2941.5	0.625	
	Standard	1872	1554.5	1701	1383.5	1469	0.3125	
	Blank	315		320		317.5		
C. vicina	1 Leg 1	9191	8873.5	10880	10562.5	9718	2.46	
	1 Leg 2	12596	12278.5	13025	12707.5	12493	3.16	2.81
	6 Legs 1	23772	23454.5	20655	20337.5	21896	5.54	
	6 Legs 2	18878	18560.5	17449	17131.5	17846	4.52	5.03
	Part leg 1	11253	10935.5	12175	11857.5	11396.5	2.88	
	Part leg 2	6861	6543.5	7258	6940.5	6742	1.71	2.30
	1 Wing 1	766	448.5	801	483.5	466	0.12	
	1 Wing 2	1266	948.5	1355	1037.5	993	0.25	0.18
	Thorax 1	43265	42947.5	58780	58462.5	50705	12.83	
	Thorax 2	45384	45066.5	55880	55562.5	50314.5	12.73	12.78
	Whole 1	37067	36749.5	49656	49338.5	43044	10.89	
	Whole 2	47338	47020.5	62180	61862.5	54441.5	13.78	12.34
C. vomitoria	1 Leg 1	6633	6315.5	6939	6621.5	6468.5	1.64	
	1 Leg 2	5253	4935.5	5179	4861.5	4898.5	1.24	1.44
	6 Legs 1	36243	35925.5	36243	35925.5	35925.5	9.09	
	6 Legs 2	30963	30645.5	32754	32436.5	31541	7.98	8.54
	Part leg 1	4136	3818.5	4125	3807.5	3813	0.97	
	Part leg 2	5357	5039.5	5179	4861.5	4950.5	1.25	1.11
	1 Wing 1	917	599.5	924	606.5	603	0.15	
	1 Wing 2	1861	1543.5	1947	1629.5	1586.5	0.40	0.28
	Thorax 1	76346	76028.5	75069	74751.5	75390	19.08	
	Thorax 2	57473	57155.5	56195	55877.5	56516.5	14.30	16.69
	Whole 1	69777	69459.5	71164	70846.5	70153	17.76	
	Whole 2	73606	73288.5	73606	73288.5	73288.5	18.55	18.15
	Burnt 1	48117	47779.5	49656	49318.5	48549	12.29	13.16

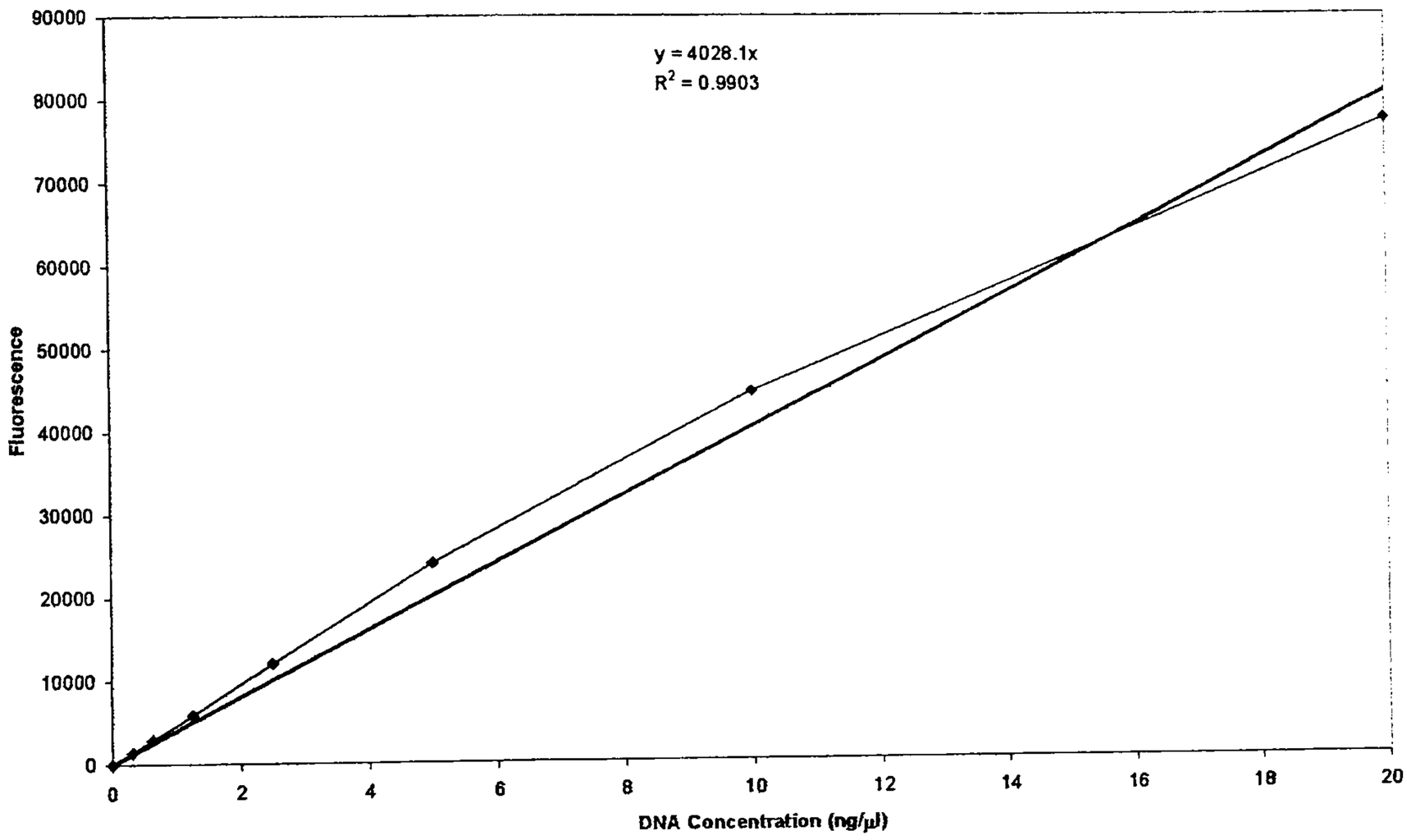
	Burnt 2	48552	48214.5	46862	46524.5	47369.5	11.99	
	Burnt 3	60117	59779.5	60797	60459.5	60119.5	15.22	
	Decomposing1	56258	55920.5	56290	55952.5	55936.5	14.16	14.02
	Decomposing2	56226	55888.5	53122	52784.5	54336.5	13.75	
	Decomposing3	44575	44237.5	44399	44061.5	44149.5	11.17	
	Decomposing4	66223	65885.5	68034	67696.5	66791	16.90	
	Decomposing5	56100	55762.5	53723	53385.5	54574	13.81	
	Decomposing6	58286	57948.5	55629	55291.5	56620	14.33	



B.

	Sample	Reading 1	Minus Blank	Reading 2	Minus Blank	Average	Concentration (ng/ml)	Mean Conc.
	Standard	76546	76216	78374	78044	77130	20	
	Standard	42422	42092	47338	47008	44550	10	
	Standard	25006	24676	23772	23442	24059	5	
	Standard	12735	12405	12039	11709	12057	2.5	
	Standard	6559	6229	5862	5532	5880.5	1.25	
	Standard	3416	3086	3079	2749	2917.5	0.625	
	Standard	1810	1480	1740	1410	1445	0.3125	
	Blank	338		322		330		
C. vicina	1 Egg	6080	5750	5796	5466	5608	1.39	1.39
	8 Eggs	25864	25534	26452	26122	25828	6.41	6.41
	L1 1	12141	11811	11904	11574	11692.5	2.90	2.90
	6 L1	27130	26800	29270	28940	27870	6.92	6.92
	L2 1	21124	20794	23375	23045	21919.5	5.44	5.89
	L2 2	25864	25534	25937	25607	25570.5	6.35	
	L3 1	32754	32424	38772	38442	35433	8.80	9.42
	L3 2	39654	39324	41948	41618	40471	10.05	
	Prepupa1	42422	42092	43878	43548	42820	10.63	10.51
	Prepupa2	40442	40112	43878	43548	41830	10.38	
	Pupa 1	51941	51611	44374	44044	47827.5	11.87	11.24
	Pupa 2	45511	45181	40556	40226	42703.5	10.60	
	Pupal case 1	1904	1574	1904	1574	1574	0.39	
	Pupal case 2	1486	1156	1503	1173	1164.5	0.29	0.58
	Pupal case 3	4590	4260	4577	4247	4253.5	1.06	
C. vomitoria	1 Egg	21605	21275	19969	19639	20457	5.08	5.08
	8 Eggs	30189	29859	29601	29271	29565	7.34	7.34
	L1 1	7017	6687	7340	7010	6848.5	1.70	1.70
	6 L1	31313	30983	31667	31337	31160	7.74	7.74
	L2 1	21544	21214	22855	22525	21869.5	5.43	5.69
	L2 2	24726	24396	23906	23576	23986	5.95	
	L3 1	32025	31695	33783	33453	32574	8.09	9.28
	L3 2	37067	36737	48009	47679	42208	10.48	
	Prepupa1	48552	48222	49101	48771	48496.5	12.04	10.96
	Prepupa2	38772	38442	41479	41149	39795.5	9.88	

	Pupa 1	44374	44044	46941	46611	45327.5	11.25	11.41
	Pupa 2	46941	46611	46941	46611	46611	11.57	
	Pupal case 1	2759	2429	2862	2532	2480.5	0.62	1.43
	Pupal case 2	9944	9614	10285	9955	9784.5	2.43	
	Pupal case 3	5342	5012	5418	5088	5050	1.25	



Appendix VII

Alignment of A+T rich region A and tRNA^{ile}, tRNA^{gln} and partial tRNA^{met} coding regions in *C. vicina* and *C. vomitoria* populations.

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                                10        20        30        40        50        60        70        80        90        100       110       120
                                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C.vicinaConsensus               TCAATATTTA TATAATTATT ATGAAGAAAT CTATATATAT ATATAAATCA TTTATTAATG TATGATAGAT ATATTTGATG TTATTATATA AATAATTTAT ATATTTATAA TTTTATCTTA
C.vicinaCheltenham1             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham2             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham3             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham4             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham5             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo1               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo2               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo3               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo4               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo5               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon1              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon2              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon3              .......... .......... .......... .......... .......... .......... .......... .......... .....C.... .......... .......... ..........
C.vicinaYork1                   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork2                   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork3                   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork4                   .......... .......... .......... .......... .......... .......G.. .......... .......... .......... .......... .......... ..........
C.vicinaNottingham1             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaKeswick1                .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaKeswick2                .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaKeswick3                .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaKeswick4                .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaDerby1                  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaGreatYarmouth1          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaGreatYarmouth2          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaGreatYarmouth3          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaGreatYarmouth4          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaGreatYarmouth5          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaPontypridd1             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaPontypridd2             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaGreenwich1              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaGreenwich2              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaStanmore1               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaStanmore2               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaStanmore3               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vomitoria Consensus           .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaMitchamJunction1     .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaMitchamJunction2     .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaMitchamJunction3     .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaMitchamJunction4     .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaMitchamJunction5     .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaMitchamJunction6     .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaMitchamJunction7     .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaHamsteadHeath1       .......... .......... .......... .......... TA........ .....C.G.. .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaHamsteadHeath2       .......... .........C .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaHamsteadHeath3       .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
C.vomitoriaHamsteadHeath4       .......... .......... .......... .......... TA........ .....C.... .......... .......... .......... ...T.....A TA...A.... ..........
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C.vomitoriaWimbledonCommon1 ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaWimbledonCommon2 ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaWimbledonCommon3 ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaWimbledonCommon4 ..... .....C ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaWimbledonCommon5 ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaStanmore1        ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaStanmore2        ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaStanmore3        ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaStanmore4        ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaStanmore5        ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaStanmore6        ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaStanmore7        ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaBoxhill1         ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaBoxhill2         ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaBoxhill3         ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaEastSheen1       ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaEastSheen2       ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaEastSheen3       ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaEastSheen4       ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaEastSheen5       ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaKeswick1         ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaKeswick2         ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaKeswick3         ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaKeswick4         ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaKeswick5         ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
C.vomitoriaYork1            ..... ..... ..... ..... TA..... .....C.... ..... ..... ..... ...T.....A TA...A.... .....
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                            130       140       150       160       170       180       190       200       210       220       230       240
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C.vicinaConsensus           TTTATGTCAT ATTTTAATAT AAGTATAAAT ATATGAATAT AATAATATAT ATTTATATAT ATATATATAT ATGCAAAATT TAATTACCTA ATCTATATAA TAAATTAAAT TAAAAATTTT
C.vicinaCheltenham1         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham2         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham3         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham4         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham5         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo1           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo2           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo3           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo4           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo5           .......... .A........ .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon1          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon2          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon3          .......... .......... .......... .........G .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork1               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork2               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork3               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork4               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaNottingham1         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaKeswick1            .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaKeswick2            .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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C.vicinaKeswick3                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaKeswick4                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaDerby1                  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaGreatYarmouth1          ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaGreatYarmouth2          ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaGreatYarmouth3          ..........  ..........  ..A.......  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaGreatYarmouth4          ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaGreatYarmouth5          ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaPontypridd1             ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaPontypridd2             ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaGreenwich1              ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaGreenwich2              ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaStanmore1               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaStanmore2               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vicinaStanmore3               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
C.vomitoria Consensus           ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaMitchamJunction1     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaMitchamJunction2     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaMitchamJunction3     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaMitchamJunction4     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaMitchamJunction5     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaMitchamJunction6     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaMitchamJunction7     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaHamsteadHeath1       ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaHamsteadHeath2       ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaHamsteadHeath3       ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaHamsteadHeath4       ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaWimbledonCommon1     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaWimbledonCommon2     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaWimbledonCommon3     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaWimbledonCommon4     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaWimbledonCommon5     ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaStanmore1            ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaStanmore2            ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaStanmore3            ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaStanmore4            ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaStanmore5            ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaStanmore6            ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaStanmore7            ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaBoxhill1             ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaBoxhill2             ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaBoxhill3             ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaEastSheen1           ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaEastSheen2           ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaEastSheen3           ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaEastSheen4           ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaEastSheen5           ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaKeswick1             ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaKeswick2             ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
C.vomitoriaKeswick3             ..........  .A........  .TA.......  ..........  ..........  ..........  ..........  ..........  ..........  .......T..  .AAA.....T  .  .......C  .......AA.
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                                250       260       270       280       290       300       310       320       330       340       350       360
                     ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C.vicinaConsensus    AATATATTT TAATTTTCAT TTACTATATT TAATTATTTA AACAATTTTA AAAACAAAAT TTTATTTTTT TTATCTACTA AAAA ATTAG TATAAAGAAA AAAAAAAAAA AAAAAACATG
C.vicinaCheltenham1  ......... ......... ......... ......... ......... ......... ......... ..C...... .... ..... ......... ......... .........
C.vicinaCheltenham2  ......... ......... ......... ......... ......... ......... ......... ..C...... .... ..... ......... ......... .........
C.vicinaCheltenham3  ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaCheltenham4  ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaCheltenham5  ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaWaterloo1    ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaWaterloo2    ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaWaterloo3    ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaWaterloo4    ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaWaterloo5    ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaWimbledon1   ......... ......... ......... ......... ......... ......... ......... ....A..A.. .... ..... ......... ......... .........
C.vicinaWimbledon2   ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaWimbledon3   ......... ......... ......... ......... ......... ......... .........
C.vicinaYork1        ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaYork2        ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaYork3        ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaYork4        ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaNottingham1  ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaKeswick1     ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaKeswick2     ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaKeswick3     ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaKeswick4     ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaDerby1       ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaGreatYarmouth1 ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaGreatYarmouth2 ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaGreatYarmouth3 ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaGreatYarmouth4 ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaGreatYarmouth5 ......... ......... ......... ......... ......... ......... ......... .....C.... .... ..... ......... ......... .........
C.vicinaPontypridd1  ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaPontypridd2  ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaGreenwich1   ......... ......... ......... ......... ......... ......... ......... .......... .... ...G. ......... ......... .........
C.vicinaGreenwich2   ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaStanmore1    ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ......... ......... .........
C.vicinaStanmore2    ......... ......... ......... ......... ......... ......... ......... .......... .... ..... ...G..... ......... .........
C.vicinaStanmore3    ......... .....C... ......... ......... ......... ......... ......... .......... .... ...G. ......... ......... .........
C.vomitoria Consensus A....A.A.. .....T.. ......... .T....... .GT..A.... ......... ......... ..T.A.TA.. ....A..... ......... ......... .........
C.vomitoriaMitchamJunction1 A....A.A.. .....T.. ......... .T....... .GT..A.... ......... ......... ..T.A.TA.. ....A..... ......... ......... .........
C.vomitoriaMitchamJunction2 A....A.A.. .....T.. ......... .T....... .GT..A.... ......... ......... ..T.A.TA.. ....A..... ......... ......... .........
C.vomitoriaMitchamJunction3 A....A.A.. .....T.. ......... .T....... .GT..A.... ......... ......... ..T.A.TA.. ....A..... ......... ......... .........
C.vomitoriaMitchamJunction4 A....A.A.. .....T.. ......... .T....... .GT..A.... ......... ......... ..T.A.TA.. ....A..... ......... ......... .........
C.vomitoriaMitchamJunction5 A....A.A.. .....T.. ......... .T....... .GT..A.... ......... ......... ..T.A.TA.. ....A..... ......... ......... .........
C.vomitoriaMitchamJunction6 A....A.A.. .....T.. ......... .T....... .GT..A.... ......... ......... ..T.A.TA.. ....A..... ......... ......... .........
C.vomitoriaMitchamJunction7 A....A.A.. .....T.. ......... .T....... .GT..A.... ......... ......... ..T.A.TA.. ....A..... ......... ......... .........
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C.vomitoriaHamsteadHeath1   A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaHamsteadHeath2   A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaHamsteadHeath3   A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaHamsteadHeath4   A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaWimbledonCommon1 A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaWimbledonCommon2 A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaWimbledonCommon3 A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaWimbledonCommon4 A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaWimbledonCommon5 A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaStanmore1        A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaStanmore2        A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaStanmore3        A....A.A.. .......T.. .......... .T........ .G...A.... .......... .......... ..T.A.TA.. ....A...G. .T........ .......... ..........
C.vomitoriaStanmore4        A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaStanmore5        A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaStanmore6        A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaStanmore7        A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaBoxhill1         A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaBoxhill2         A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaBoxhill3         A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaEastSheen1       A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaEastSheen2       A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaEastSheen3       A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaEastSheen4       A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaEastSheen5       A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaKeswick1         A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaKeswick2         A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaKeswick3         A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaKeswick4         A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ......
C.vomitoriaKeswick5         A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........
C.vomitoriaYork1            A....A.A.. .......T.. .......... .T........ .GT..A.... .......... .......... ..T.A.TA.. ....A..... .......... .......... ..........

                                    370        380        390        400        410        420        430        440        450        460        470        480
                            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C.vicinaConsensus           AAAACCCTAT TCTTAATGAA TTGCCTGATA AAAAGGATTA CCTTGATAGG GTAAATCATG TAATAATATT ACATTCATTA TTATATTTAA TAGAATTAAA CTATTTCTAA AAGTATCAAA
C.vicinaCheltenham1         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham2         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham3         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham4         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaCheltenham5         .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo1           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo2           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo3           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo4           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWaterloo5           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon1          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon2          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaWimbledon3
C.vicinaYork1               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork2               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C.vicinaYork3               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
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C.vicinaYork4 ....
C.vicinaNottingham1 ....
C.vicinaKeswick1 ....
C.vicinaKeswick2 ....
C.vicinaKeswick3 ....
C.vicinaKeswick4 ....
C.vicinaDerby1 ....
C.vicinaGreatYarmouth1 ....
C.vicinaGreatYarmouth2 ....
C.vicinaGreatYarmouth3 ....
C.vicinaGreatYarmouth4 ....
C.vicinaGreatYarmouth5 ....
C.vicinaPontypridd1 ....
C.vicinaPontypridd2 ....
C.vicinaGreenwich1 ....
C.vicinaGreenwich2 ....
C.vicinaStanmore1 ....
C.vicinaStanmore2 ....
C.vicinaStanmore3 ....
C.vomitoria Consensus ....
C.vomitoriaMitchamJunction1 ....
C.vomitoriaMitchamJunction2 ....
C.vomitoriaMitchamJunction3 ....
C.vomitoriaMitchamJunction4 ....
C.vomitoriaMitchamJunction5 ....
C.vomitoriaMitchamJunction6 ....
C.vomitoriaMitchamJunction7 ....
C.vomitoriaHamsteadHeath1 ....
C.vomitoriaHamsteadHeath2 ....
C.vomitoriaHamsteadHeath3 ....
C.vomitoriaHamsteadHeath4 ....
C.vomitoriaWimbledonCommon1 ....
C.vomitoriaWimbledonCommon2 ....
C.vomitoriaWimbledonCommon3 ....
C.vomitoriaWimbledonCommon4 ....
C.vomitoriaWimbledonCommon5 ....
C.vomitoriaStanmore1 ....
C.vomitoriaStanmore2 ....
C.vomitoriaStanmore3 ....
C.vomitoriaStanmore4 ....
C.vomitoriaStanmore5 ....
C.vomitoriaStanmore6 ....
C.vomitoriaStanmore7 ....
C.vomitoriaBoxhill1 ....
C.vomitoriaBoxhill2 ....
C.vomitoriaBoxhill3 ....
C.vomitoriaEastSheen1 ....
C.vomitoriaEastSheen2 ....
C.vomitoriaEastSheen3 ....
C.vomitoriaEastSheen4 ....

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C.vomitoriaEastSheen5  .......... .......... .......... .......... .......... .......... .......... ..........  ..... ..... ..... .....
C.vomitoriaKeswick1    .......... .......... .......... .......... .......... .......... .......... ..........  ..... ..... ..... .....
C.vomitoriaKeswick2    .......... .......... .......... .......... .......... .......... .......... ..........  ..... ..... ..... .....
C.vomitoriaKeswick3    .......... .......... .......... .......... .......... .......... .......... ..........  ..... ..... ..... .....
C.vomitoriaKeswick4
C.vomitoriaKeswick5    .......... .......... .......... .......... .......... .......... .......... ..........  ..... ..... ..... .....
C.vomitoriaYork1       .......... .......... .......... .......... .......... .......... .......... ..........  ..... ..... ..... .....
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                                490         500         510         520
                       ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
C.vicinaConsensus      AACTTTTGTG CATCATACAC CAAAATATAT TTATAATTAA AAAGAT
C.vicinaCheltenham1    .......... .......... .......... .......... ......
C.vicinaCheltenham2    .......... .......... .......... .......... ......
C.vicinaCheltenham3    .......... .......... .......... .......... ......
C.vicinaCheltenham4    .......... .......... .......... .......... ......
C.vicinaCheltenham5    .......... .......... .......... .......... ......
C.vicinaWaterloo1      .......... .......... .......... .......... ......
C.vicinaWaterloo2      .......... .......... .......... .......... ......
C.vicinaWaterloo3      .......... .......... .......... .......... ......
C.vicinaWaterloo4
C.vicinaWaterloo5      .......... .......... .......... .......... ......
C.vicinaWimbledon1     .......... .......... .......... .......... ......
C.vicinaWimbledon2     .......... .......... .......... .......... ......
C.vicinaWimbledon3
C.vicinaYork1          .......... .......... .......... .......... ......
C.vicinaYork2          .......... .......... .......... .......... ......
C.vicinaYork3          .......... .......... .......... .......... ......
C.vicinaYork4          .......... .......... .......... .......... ......
C.vicinaNottingham1    .......... .......... .......... .......... ......
C.vicinaKeswick1       .......... .......... .......... .......... ......
C.vicinaKeswick2
C.vicinaKeswick3       .......... .......... .......... .......... ......
C.vicinaKeswick4       .......... .......... .......... .......... ......
C.vicinaDerby1         .......... .......... .......... .......... ......
C.vicinaGreatYarmouth1 .......... .......... .......... .......... ......
C.vicinaGreatYarmouth2 .......... .......... .......... .......... ......
C.vicinaGreatYarmouth3
C.vicinaGreatYarmouth4 .......... .......
C.vicinaGreatYarmouth5 .......... .......... .......... .......... ......
C.vicinaPontypridd1    .......... .......... .......... .......... ......
C.vicinaPontypridd2    .......... .......... .......... .......... ......
C.vicinaGreenwich1     .......... .......... .......... .......... ......
C.vicinaGreenwich2     .......... .......... .......... .......... ......
C.vicinaStanmore1      .......... .......... .......... .......... ......
C.vicinaStanmore2      .......... .......... .......... .......... ......
C.vicinaStanmore3      .......... .......... .......... .......... ......
C.vomitoria Consensus  .......... .......... .........  A....T.... ......
C.vomitoriaMitchamJunction1 ..... .......... .........  A....T.... ......
C.vomitoriaMitchamJunction2
C.vomitoriaMitchamJunction3 ..... .......... .........  A....T.... ......
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C.vomitoriaMitchamJunction4  ..........  ..........  .........  A....T....  ......
C.vomitoriaMitchamJunction5  ..........  ..........  .........  A....T....  ......
C.vomitoriaMitchamJunction6  ..........  ..........  .........  A....T.C..  ......
C.vomitoriaMitchamJunction7  ..........  ..........  .........  A....T....  ......
C.vomitoriaHamsteadHeath1    ..........  ..........  .........  A....T....  ......
C.vomitoriaHamsteadHeath2    ..........  ..........  .........  A....T..
C.vomitoriaHamsteadHeath3    ..........  ..........  .........  A....T.C..  ......
C.vomitoriaHamsteadHeath4    ..........  ..........  .........  A....T..
C.vomitoriaWimbledonCommon1  ..........  ..........  .........  A....T.C
C.vomitoriaWimbledonCommon2  ..........  ..........  .........  A....T....  ......
C.vomitoriaWimbledonCommon3  ..........  ..........  .........  A....T.C..  ......
C.vomitoriaWimbledonCommon4  ..........  ..........  .........  A....T....  ......
C.vomitoriaWimbledonCommon5  ..........  .........
C.vomitoriaStanmore1         ..........  ..........  .........  A....
C.vomitoriaStanmore2         ..........  ..........  .........  A....T....  ......
C.vomitoriaStanmore3         ..........  ..........  .........  A....T....  ......
C.vomitoriaStanmore4         ..........  ..........  .........  A....T....  ......
C.vomitoriaStanmore5         ..........  ..........  .........  A....T....  ......
C.vomitoriaStanmore6         ..........  ..........  .....
C.vomitoriaStanmore7         ..........  ..........  .........  A....T....  ......
C.vomitoriaBoxhill1          ..........  ..........  .........  A....T....  ......
C.vomitoriaBoxhill2          ..........  ..........  .........  A....T.C..  ......
C.vomitoriaBoxhill3          ..........  ..........  .........  A....T....  ......
C.vomitoriaEastSheen1        ..........  ..........  .........  A....T....  ......
C.vomitoriaEastSheen2        ..........  ..........  .........  A....T....  ......
C.vomitoriaEastSheen3        ..........  .........
C.vomitoriaEastSheen4
C.vomitoriaEastSheen5        ..........  ..........  .........  A....T....  ......
C.vomitoriaKeswick1          ..........  ..........  .........  A....T....  ......
C.vomitoriaKeswick2          ..........  ..........  .........  A....T....  ......
C.vomitoriaKeswick3          ..........  ..........  .........  A....T....  ......
C.vomitoriaKeswick4
C.vomitoriaKeswick5          ..........  ..........  .........  A....T....  ......
C.vomitoriaYork1             ..........  ..........  .........  A....T....  ......
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Allele frequencies for *C. vicina* populations of 12 loci showing intraspecific variation. Bold values indicate private alleles.

Nucleotide Position	Nucleotide	Cheltenham	Waterloo	Wimbledon	York	Nottingham	Keswick	Derby	Great Yarmouth	Pontypridd	Greenwich	Stanmore
58	A	1.000	1.000	1.000	0.750	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	G	0.000	0.000	0.000	**0.250**	0.000	0.000	0.000	0.000	0.000	0.000	0.000
96	T	1.000	1.000	0.667	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.000	**0.333**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
132	T	1.000	0.800	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	A	0.000	**0.200**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
143	G	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.800	1.000	1.000	1.000
	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.200**	0.000	0.000	0.000
160	T	1.000	1.000	0.667	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	G	0.000	0.000	**0.333**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
258	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.667
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.333**
312	A	0.600	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	C	**0.400**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
314	C	1.000	1.000	0.667	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	A	0.000	0.000	**0.333**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
316	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.800	1.000	1.000	1.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.200**	0.000	0.000	0.000
317	C	1.000	1.000	0.667	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	A	0.000	0.000	**0.333**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
327	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.500	0.667
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.333
342	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.667
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.333**

Allele frequencies for *C. vomitoria* populations of 4 loci showing intraspecific variation. Bold values indicate private alleles.

Nucleotide Position	Nucleotide	Mitcham Junction	Hampstead Heath	Wimbledon Common	Stanmore	Box Hill	East Sheen	Keswick	York
20	T	1.000	0.750	0.800	1.000	1.000	1.000	1.000	1.000
	C	0.000	0.250	0.200	0.000	0.000	0.000	0.000	0.000
58	A	1.000	0.750	1.000	1.000	1.000	1.000	1.000	1.000
	G	0.000	**0.250**	0.000	0.000	0.000	0.000	0.000	0.000
327	A	1.000	1.000	1.000	0.857	1.000	1.000	1.000	1.000
	G	0.000	0.000	0.000	**0.143**	0.000	0.000	0.000	0.000
330	A	1.000	1.000	1.000	0.857	1.000	1.000	1.000	1.000
	T	0.000	0.000	0.000	**0.143**	0.000	0.000	0.000	0.000

Appendix VIII.

Alignment of A+T rich region C for *C. vicina* and *C. vomitoria* populations. Species are aligned to the respective consensus sequence. Dot indicates similar nucleotide to the consensus.

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                                  10        20        30        40        50        60        70        80        90
                                  |---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-
Consensus C. vicina               ACAATAAAATTTAAAAATATAATTTTTTTTAACTTATAAATTTAACATTATTTAAATAAATAAAATTAGTAAACATGAAAACCCTATTCTT
C. vicina Cheltenham 1            ..................................................................................
C. vicina Cheltenham 2            ..................................................................................
C. vicina Cheltenham 3            ..................................................................................
C. vicina Cheltenham 4            ..................................................................................
C. vicina Wimbledon 1             ..................................................................................
C. vicina Wimbledon 2             .......T..................T.....................................................
C. vicina Wimbledon 3             ........................T.....................................................
C. vicina Wimbledon 4             ..................................................................................
C. vicina Wimbledon 5             .......T..................T.....................................................
C. vicina Nottingham 1            ..................................................................................
C. vicina Nottingham 2            ...................................................................A..............
C. vicina Nottingham 3            ..................................................................................
C. vicina Greenwich 1             ..................................................................................
C. vicina Greenwich 2             ..................................................................................
C. vicina Mitcham Junction 1      .......T..................T.....................................................
C. vicina Mitcham Junction 2      .......T..................................................................
C. vicina Mitcham Junction 3      .......T..................................................................
C. vicina York 1                  ..................................................................................
C. vicina York 2                  ..................................................................................
C. vicina York 3                  ..................................................................................
C. vicina York 4                  ..................................................................................
C. vicina Nonsuch Park 1          ........................T.....................................A..............
C. vicina Nonsuch Park 2          ..................................................................................
C. vicina Nonsuch Park 3          ...................................................................A..............
C. vicina Waterloo 1              ..................................................................................
C. vicina Waterloo 2              ..................................................................................
C. vicina Waterloo 3              ..................................................................................
C. vicina Pontypridd 1            ..................................................................................
C. vicina Pontypridd 2            ..................................................................................
C. vicina Snaresbrook 1           ..................................................................................
C. vicina Snaresbrook 2           ..................................................................................
C. vicina Keswick 1               ..................................................................................
C. vicina Keswick 2               ...................................................................A..............
C. vicina Keswick 3               ..................................................................................
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                                10        20        30        40        50        60        70        80        90
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Consensus C. vomitoria  TACAATTAATTTAAAATTTATAAATTTTTTAATTTATTAATATAATATTATT AAATAAATTAAAATTAGTAAACATGAAAACCCTATTCTT
C. vomitoria Cheltenham 1 ..................................................... .....................................
C. vomitoria 1          ..................................................... .....................................
C. vomitoria 2          ..................................................... .....................................
C. vomitoria 3          ..................................................... .....................................
C. vomitoria 4          ..........................A.......................... ..............G......................
C. vomitoria 5          ....................T.......................................... .....T.....................
C. vomitoria Pett Wood 1 ..................................................... .....................................
C. vomitoria Pett Wood 2 ..................................................... .....................................
C. vomitoria Pett Wood 3 ....................T.......................................... .....T.....................
C. vomitoria Keswick 1   ..........................A.......................... ........G....A.....................
C. vomitoria Keswick 2   ..........................A.......................... ..............G......................
C. vomitoria Keswick 3   ..........................A.......................... ........G....A.....................
C. vomitoria Keswick 4   ..........................A.......................... ........G....A.....................
C. vomitoria Stanmore 1  ..........................A.......................... ..............G.T...................
C. vomitoria Stanmore 2  ..................................................... .....................................
C. vomitoria Mitcham Junction ..........................A.......................... ..............G......................
C. vomitoria Mitcham Junction ..................................................... .....................................
C. vomitoria Mitcham Junction ..................................................... .....................................
C. vomitoria Nonsuch Park 1 ..........................A.......................... ..............G......................
C. vomitoria Nonsuch Park 2 ..................................................... .....................................
C. vomitoria Nonsuch Park 3 ..................................................... .....................................
C. vomitoria Wimbledon Common ..........................A.......................... ..............G......................
C. vomitoria Wimbledon Common ..................................................... .....................................
C. vomitoria Wimbledon Common ..................................................... .....................................
C. vomitoria Wimbledon Common ..........................A.......................... ..............G.T...................
C. vomitoria Box hill 1   ....................T.......................................... .....T.....................
C. vomitoria Box hill 2   ..................................................... .....................................
C. vomitoria Box hill 3   ..................................................... .....................................
C. vomitoria East Sheen 1 ..................................................... .....................................
C. vomitoria East Sheen 2 ....................T.......................................... .....T.....................
C. vomitoria Hampstead Heath 1 ..................................................... .....................................
C. vomitoria Hampstead Heath 2 ..........................A.......................... ..............G.T...................
```

Allele frequencies for intraspecific variation in A+T rich region C in *C. vicina* populations. Values in bold font indicate private alleles.

Nucleotide Position	Nucleotide	Cheltenham	Wimbledon	Nottingham	Greenwich	Mitcham Junction	York	Nonsuch Park	Waterloo	Pontypridd	Snaresbrook	Keswick
8	A	1.000	0.600	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000
	T	0.000	0.400	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
30	A	1.000	0.400	1.000	1.000	0.667	1.000	0.667	1.000	1.000	1.000	1.000
	T	0.000	0.600	0.000	0.000	0.333	0.000	0.333	0.000	0.000	0.000	0.000
66	T	1.000	1.000	0.667	1.000	1.000	1.000	0.333	1.000	1.000	1.000	0.667
	A	0.000	0.000	0.333	0.000	0.000	0.000	0.667	0.000	0.000	0.000	0.333

Allele frequencies for intraspecific variation in A+T rich region C in *C. vomitoria* populations. Values in bold font indicate private alleles

Nucleotide Position	Nucleotide	Cheltenham	Commercial	Petts Wood	Keswick	Stanmore	Mitcham Junction	Nonsuch Park	Wimbledon Common	Box Hill	East Sheen	Hampstead Heath
22	A	1.000	0.800	0.667	1.000	1.000	1.000	1.000	1.000	0.667	0.500	1.000
	T	0.000	0.200	0.333	0.000	0.000	0.000	0.000	0.000	0.333	0.500	0.000
38	T	1.000	0.800	1.000	0.000	0.500	0.667	0.667	0.500	1.000	1.000	0.500
	A	0.000	0.200	0.000	1.000	0.500	0.333	0.333	0.500	0.000	0.000	0.500
58	A	1.000	0.800	0.667	1.000	1.000	1.000	1.000	1.000	0.667	0.500	1.000
	T	0.000	0.200	0.333	0.000	0.000	0.000	0.000	0.000	0.333	0.500	0.000
61	T	1.000	1.000	1.000	0.250	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	G	0.000	0.000	0.000	**0.750**	0.000	0.000	0.000	0.000	0.000	0.000	0.000
66	T	1.000	0.800	1.000	0.000	0.500	0.667	0.667	0.500	1.000	1.000	0.500
	G	0.000	0.200	0.000	0.250	0.500	0.333	0.333	0.500	0.000	0.000	0.500
	A	0.000	0.000	0.000	**0.750**	0.000	0.000	0.000	0.000	0.000	0.000	0.000
68	G	1.000	1.000	1.000	1.000	0.500	1.000	1.000	0.750	1.000	1.000	0.500
	T	0.000	0.000	0.000	0.000	0.500	0.000	0.000	0.250	0.000	0.000	0.500

Appendix IX.

Absorbance readings (260nm) for individual *C. vicina* pupal samples of different ages (given in ADH). Colours assessed by observation.

	ADH	Sample.	A_{260}	Conc (ng/μl)		ADH	Sample.	A_{260}	Conc (ng/μl)
Experiment 1	2880 Larvae	1	1.064	2128	Experiment 2	3360 white/ peach	1	0.449	898
		2	0.879	1758			2	0.607	1214
		3	0.991	1982			3	0.783	1566
		4	1.849	3698			4	0.599	1198
		5	1.078	2156			5	0.400	800
		6	1.068	2136		3840 orange/ red	1	0.360	720
		7	0.390	780			2	0.279	558
		8	0.918	1836			3	0.488	976
		9	1.012	2024			4	0.207	414
		10	1.108	2216			5	0.268	536
		11	1.250	2500			6	0.226	452
		12	0.717	1434			7	0.236	472
		13	0.918	1836			8	0.227	454
		14	0.512	1024			9	0.264	528
	3360 white/ orange	1	0.993	1986		4320 red	1	0.339	678
		2	0.807	1614			2	0.600	1200
		3	0.647	1294			3	0.240	480
		4	0.481	962			4	0.273	546
	3840 orange/ red	1	1.065	2130			5	0.275	550
		2	0.432	864			6	0.255	510
		3	1.203	2406			7	0.231	462
		4	0.741	1482			8	0.348	696
		5	0.751	1502		4800 red	1	0.188	376
		6	0.951	1902			2	0.166	332
		7	1.016	2032			3	0.269	538
		8	0.680	1360			4	0.214	428
		9	0.913	1826			5	0.368	736
	4320 red	1	0.910	1820			6	0.294	588
		2	0.763	1526			7	0.771	1542
		3	0.902	1804			8	0.607	1214
		4	0.466	932		5280 red	1	0.288	576
		5	1.125	2250			2	0.380	760
		6	0.472	944			3	0.276	552
		7	0.949	1898			4	0.201	402
		8	0.625	1250			5	0.348	696
		9	0.434	868			6	0.310	620
		10	0.634	1268			7	0.178	356
		11	0.800	1600			8	0.388	776
		12	0.804	1608			9	0.111	222
		13	0.667	1334			10	0.331	662

		14	0.998	1996
		15	1.085	2170
	4800 red	1	0.845	1690
		2	0.729	1458
		3	0.719	1438
		4	0.806	1612
		5	0.523	1046
		6	0.563	1126
		7	0.579	1158
		8	0.813	1626
		9	1.031	2062
		10	0.680	1360
		11	0.530	1060
		12	0.917	1834
		13	0.714	1428
		14	0.747	1494
		15	0.808	1616
		16	0.768	1536
		17	0.500	1000
		18	1.006	2012
		19	0.269	538
		20	0.308	616
	5760 red	1	0.213	426
		2	2.000	4000
		3	0.226	452
		4	0.200	400
		5	0.350	700
		6	0.613	1226
		7	0.412	824
		8	0.511	1022
		9	0.385	770
		10	0.456	912
		11	0.586	1172
		12	0.555	1110
	6240 red/ drk red	1	0.686	1372
		2	1.347	2694
		3	0.545	1090
		4	1.150	2300
		5	0.704	1408
		6	0.991	1982
		7	0.707	1414
		8	0.841	1682
		9	0.727	1454
		10	0.734	1468
		11	0.921	1842
		12	0.785	1570
		13	0.709	1418
		14	0.928	1856
		15	0.625	1250
		11	0.431	862
		12	0.238	476
		13	0.236	472
		14	0.230	460
		15	0.323	646
	5760 red	1	0.260	520
		2	0.397	794
		3	0.319	638
		4	0.227	454
		5	0.292	584
		6	0.391	782
		7	0.696	1392
		8	0.228	456
		9	0.205	410
		10	0.191	382
		11	0.260	520
		12	0.423	846
		13	0.281	562
		14	0.416	832
		15	0.197	394
		16	0.114	228
	6240 red/ drk red	1	0.386	772
		2	0.227	454
		3	0.307	614
		4	0.441	882
		5	0.254	508
		6	0.191	382
		7	0.122	244
		8	0.168	336
		9	0.105	210
		10	0.120	240
		11	0.382	764
		12	0.416	832
		13	0.284	568
		14	0.194	388
		15	0.103	206
		16	0.124	248
		17	0.286	572
		18	0.302	604
		19	0.364	728
		20	0.350	700
		21	0.161	322
		22	0.108	216
		23	0.164	328
		24	0.623	1246
		25	0.227	454
		26	0.473	946
	6720 red/	1	0.597	1194
		2	0.292	584

		16	0.739	1478
		17	0.903	1806
		18	0.711	1422
		19	0.876	1752
		20	0.456	912
		21	0.935	1870
		22	0.793	1586
		23	1.147	2294
		24	0.811	1622
		25	0.580	1160
		26	1.106	2212
		27	0.782	1564
		28	1.025	2050
		29	0.814	1628
		30	0.644	1288
		31	0.750	1500
		32	0.655	1310
		33	1.503	3006
		34	0.905	1810
		35	1.037	2074
		36	0.765	1530
		37	0.833	833
		38	0.319	638
		39	0.173	346
		40	0.353	706
		41	0.397	794
		42	0.280	560
		43	0.184	368
		44	0.387	774
		45	0.460	920
	6720 red/ drk red	1	0.512	1024
		2	0.386	772
		3	0.200	400
		4	0.215	430
		5	0.245	490
		6	0.118	236
		7	0.932	1864
		8	0.740	1480
		9	0.453	906
		10	0.353	706
		11	0.389	778
		12	0.552	1104
		13	0.462	924
		14	0.893	1786
		15	0.860	1720
	7200 drk red	1	0.632	1264
		2	0.552	1104
		3	0.494	988
		4	1.973	3946
		3	0.135	270
		4	0.548	1096
		5	0.431	862
		6	0.299	598
		7	0.565	1130
		8	0.260	520
		9	0.269	538
		10	0.551	1102
		11	0.289	578
		12	0.249	498
		13	0.952	1904
		14	0.283	566
	7200 red/ drk red	1	0.359	718
		2	0.248	496
		3	0.417	834
		4	0.320	640
		5	0.411	822
		6	0.351	702
		7	0.727	1454
		8	0.527	1054
		9	0.311	622
		10	0.235	470
		11	0.355	710
		12	0.249	498
		13	0.368	736
		14	0.265	530
		15	0.260	520
		16	0.238	476
		17	0.351	702
		18	0.567	1134
		19	0.217	434
		20	0.281	562
		21	0.406	812
		22	0.338	676
		23	0.317	634
		24	0.413	826
		25	0.610	1220
		26	0.246	492
		27	0.316	632
		28	0.482	964
		29	0.237	474
		30	0.341	682
	7680 drk red	1	0.558	1116
		2	0.455	910
		3	0.200	400
		4	0.412	824
		5	0.386	772
		6	0.308	616
		7	0.232	464

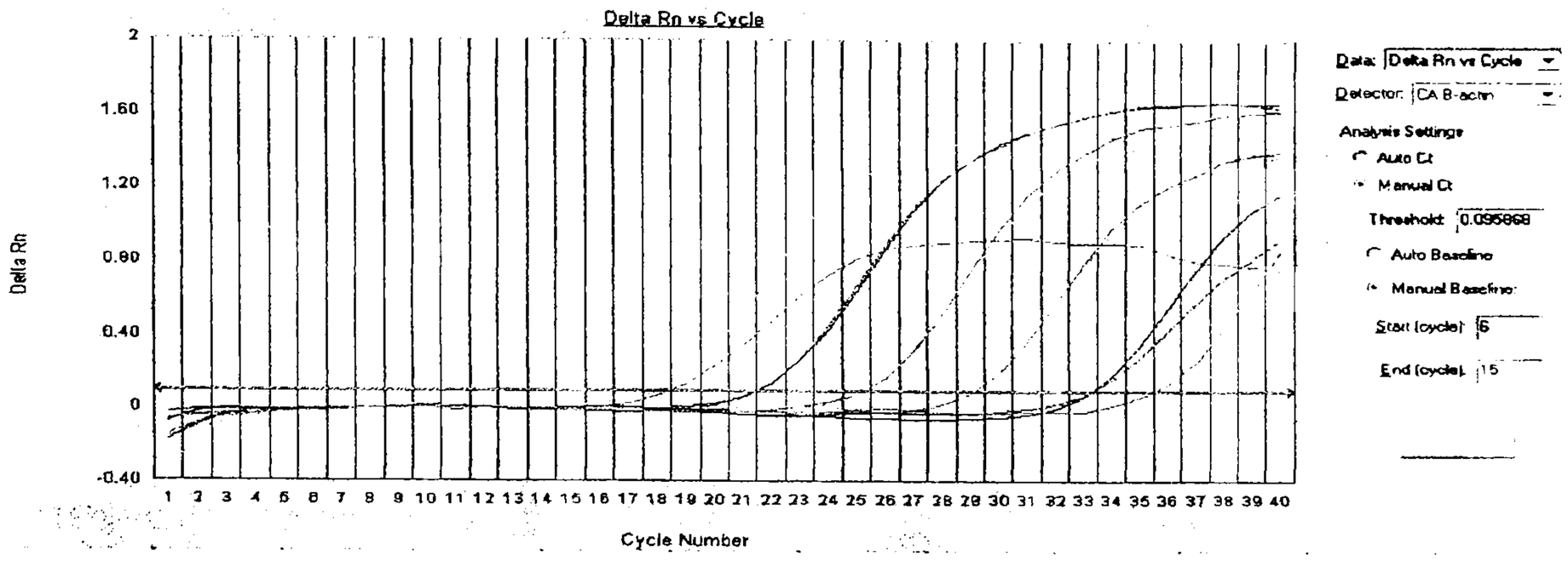
		5	0.708	1416
		6	0.303	606
		7	0.855	1710
		8	0.505	1010
		9	0.777	1554
		10	0.570	1140
		11	0.236	1416
		12	0.529	1058
		13	0.739	1478
	7680 drk red	1	0.276	552
		2	0.970	1940
		3	0.789	1578
		4	0.585	1170
		5	0.586	1172
		6	0.714	1428
		7	0.707	1414
		8	0.476	952
		9	0.524	1048
		10	0.425	850
		11	0.585	1170
		12	0.565	1130
		13	0.546	1092
		14	0.772	1544
		15	0.598	1196
		16	0.594	1188
		17	0.587	1174
		18	0.676	1352
		19	0.382	764
		20	0.682	1364
		21	0.472	944
		22	0.349	698
		23	0.619	1238
		24	0.784	1568
		25	0.581	1162
		26	0.538	1076
		27	0.611	1222
		28	0.544	1088
		29	0.705	1410
		30	0.472	944
	8640 drk red	1	0.672	1344
		2	0.783	1566
		3	0.413	826
		4	1.451	2902
		5	0.927	1854
		6	0.633	1266
		7	1.166	2332
	9120 drk red	1	0.774	1548
		2	0.362	724
		3	0.246	492
		8	0.361	722
		9	0.429	858
		10	0.286	572
	8160 drk red	1	0.425	850
		2	0.391	782
		3	0.219	438
		4	0.247	494
		5	0.351	702
		6	0.336	672
		7	0.389	778
		8	0.237	474
		9	0.330	660
		10	0.209	418
		11	0.204	408
		12	0.479	958
		13	0.372	744
	8640 drk red	1	0.305	610
		2	0.462	924
		3	0.379	758
		4	0.442	884
		5	0.389	778
		6	0.648	1296
		7	0.449	898
		8	0.601	1202
		9	0.249	498
		10	0.165	330
		11	0.365	730
		12	0.268	536
		13	0.420	840
		14	0.494	988
		15	0.292	584
		16	0.580	1160
	9120 v.drk red	1	0.321	642
		2	0.160	320
		3	0.106	212
		4	0.129	258
		5	0.154	308
		6	0.161	322
		7	0.123	246
		8	0.094	188
		9	0.100	200
		10	0.337	674
		11	0.228	456
		12	0.173	346
		13	0.351	702
	9600 v.drk red	1	0.365	730
		2	0.483	966
		3	0.249	498
		4	0.370	740

		4	0.281	562
		5	1.301	2602
		6	0.930	1860
		7	0.839	1678
		8	1.055	2110
		9	0.910	1820
		10	1.463	2926
		11	0.929	1858
		12	0.660	1320
		13	0.744	1488
		14	0.822	1644
		15	0.908	1816
		16	0.488	976
	9600 v.drk red	1	0.466	932
		2	0.497	994
		3	0.212	424
		4	0.366	732
		5	0.572	1144
		6	0.327	654
		7	0.178	356
		8	0.391	782
		9	0.242	484
	10080 v.drk red	1	0.957	1914
		2	0.630	1260
		3	0.722	1444
		4	0.828	1656
	10560 v.drk red	1	0.487	974
		2	0.271	542
		3	0.301	602
		4	0.443	886
		5	0.558	1116
		6	0.541	1082
		7	0.439	878
		8	0.667	1334
		9	0.420	840
		10	0.501	1002
		11	0.690	1380
		12	0.893	1786
		13	0.572	1144
		14	0.408	816
		15	0.529	1058
		16	0.602	1204
		17	0.632	1264
		5	0.272	544
		6	0.459	918
		7	0.483	966
		8	0.297	594
		9	0.301	602
		10	0.036	72
		11	0.197	394
		12	0.626	1252
		13	0.245	490
		14	0.443	886
		15	0.133	266
	10080 v.drk red	1	0.181	362
		2	0.203	406
		3	0.207	414
		4	0.300	600
		5	0.183	366
		6	0.414	828
		7	0.558	1116
		8	0.530	1060
		9	0.175	350
		10	0.427	854
		11	0.758	1516
		12	0.154	308
		13	0.415	830
		14	0.485	970
		15	0.162	324
	11040 v.drk red	1	0.082	164
		2	0.052	104
		3	0.072	144
		4	0.589	1178
		5	0.169	338

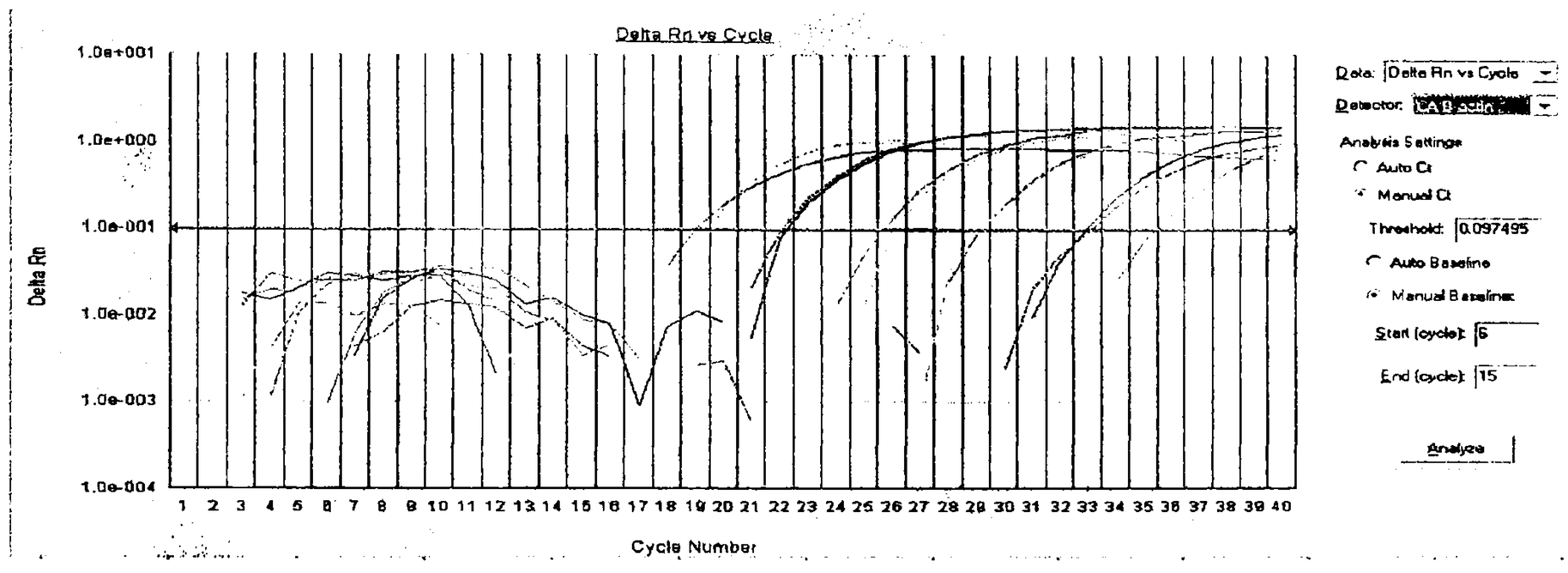
Appendix X.

Real time amplification plots to examine primer and probe efficiency.

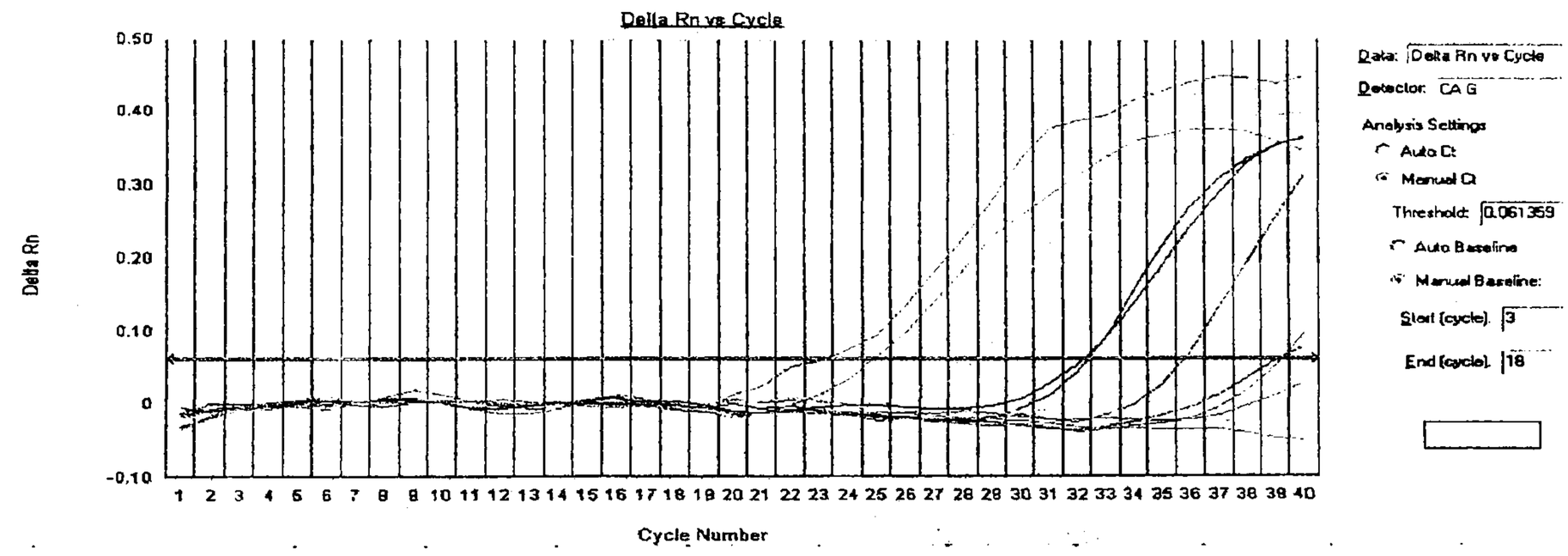
Actin – linear view



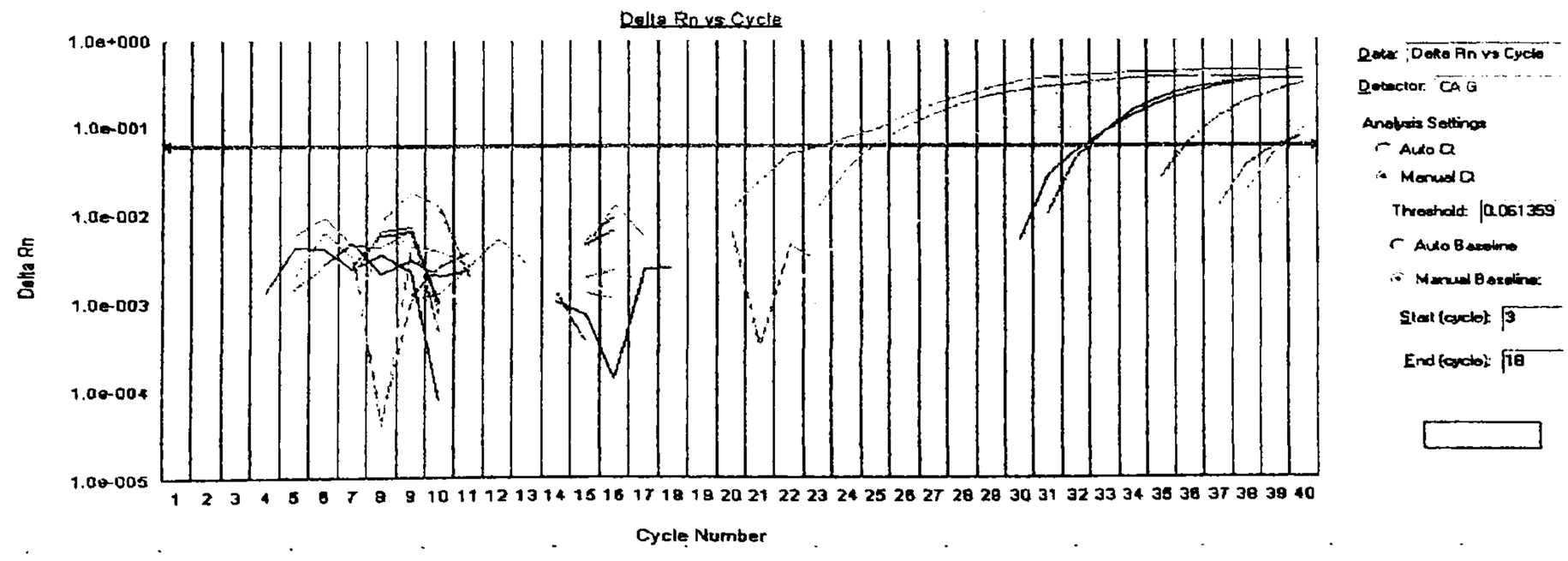
log view



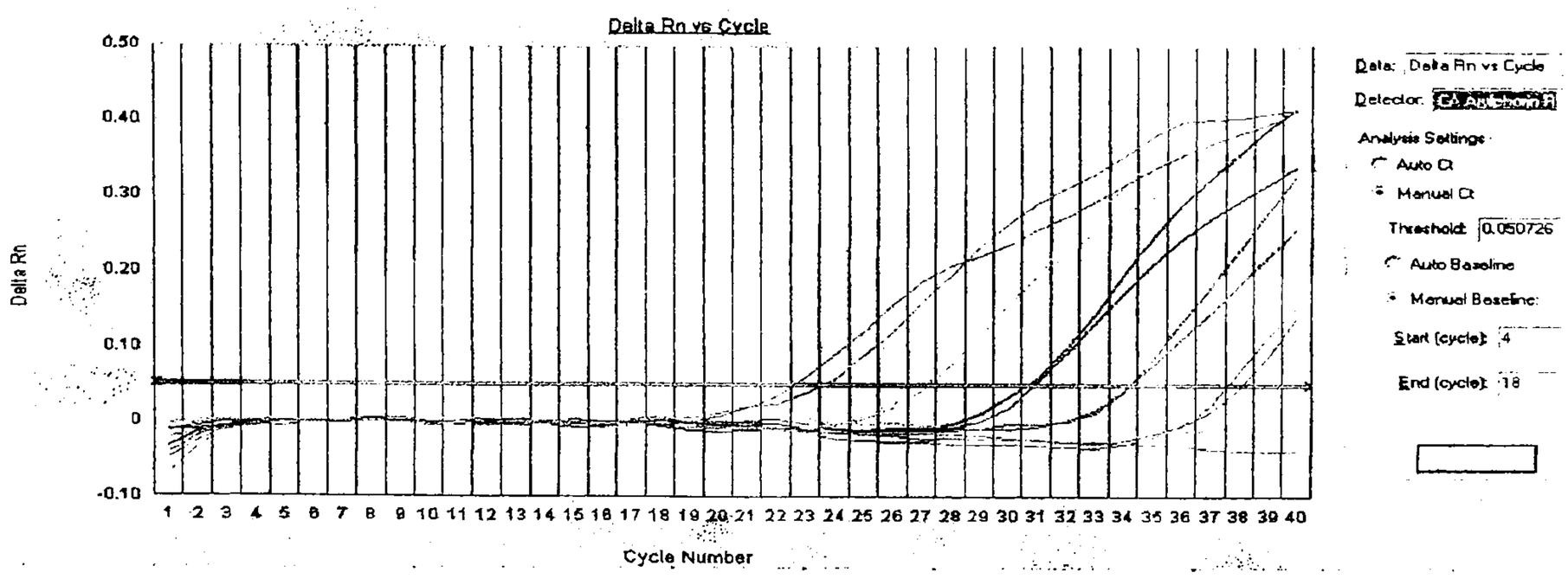
Primer G - linear view



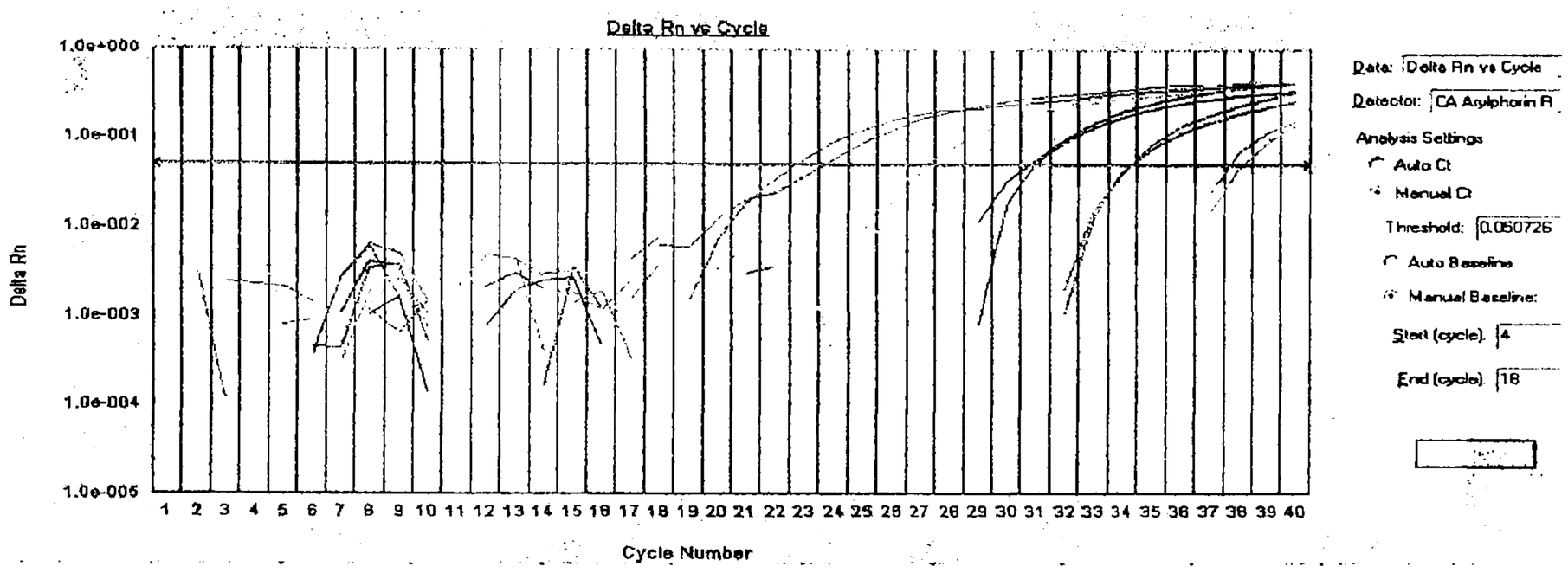
log view



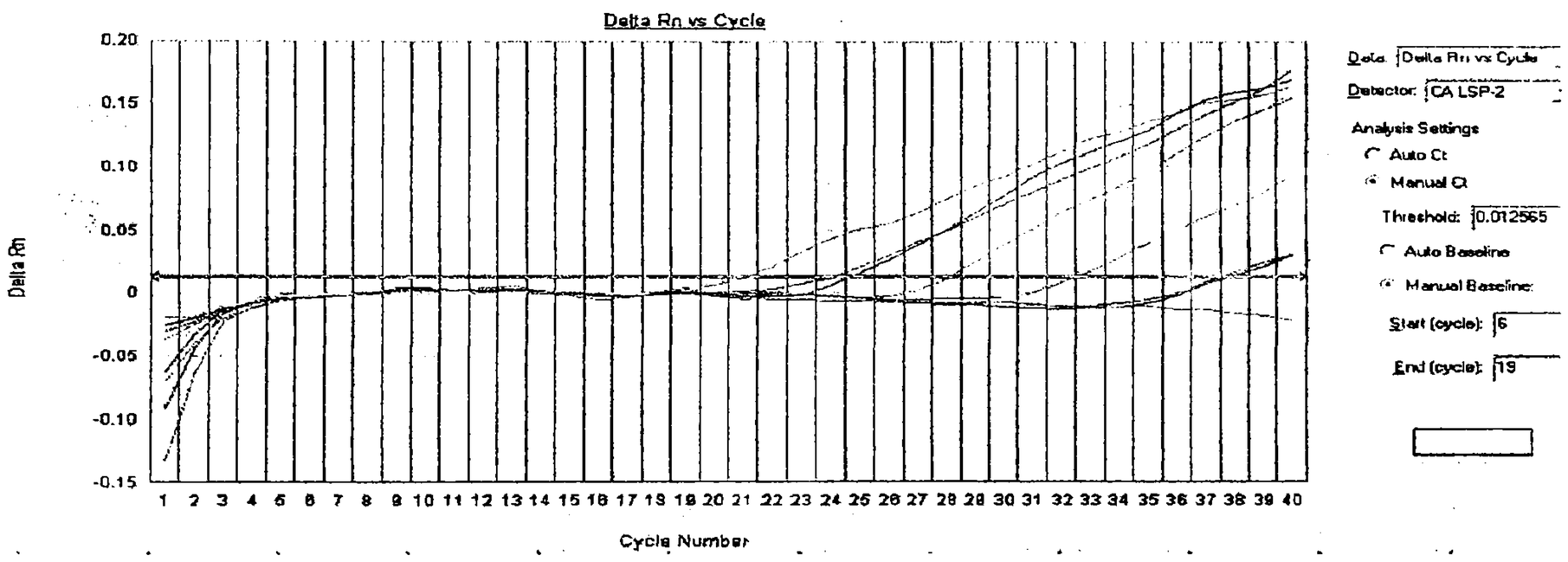
Arylphorin Receptor - linear view



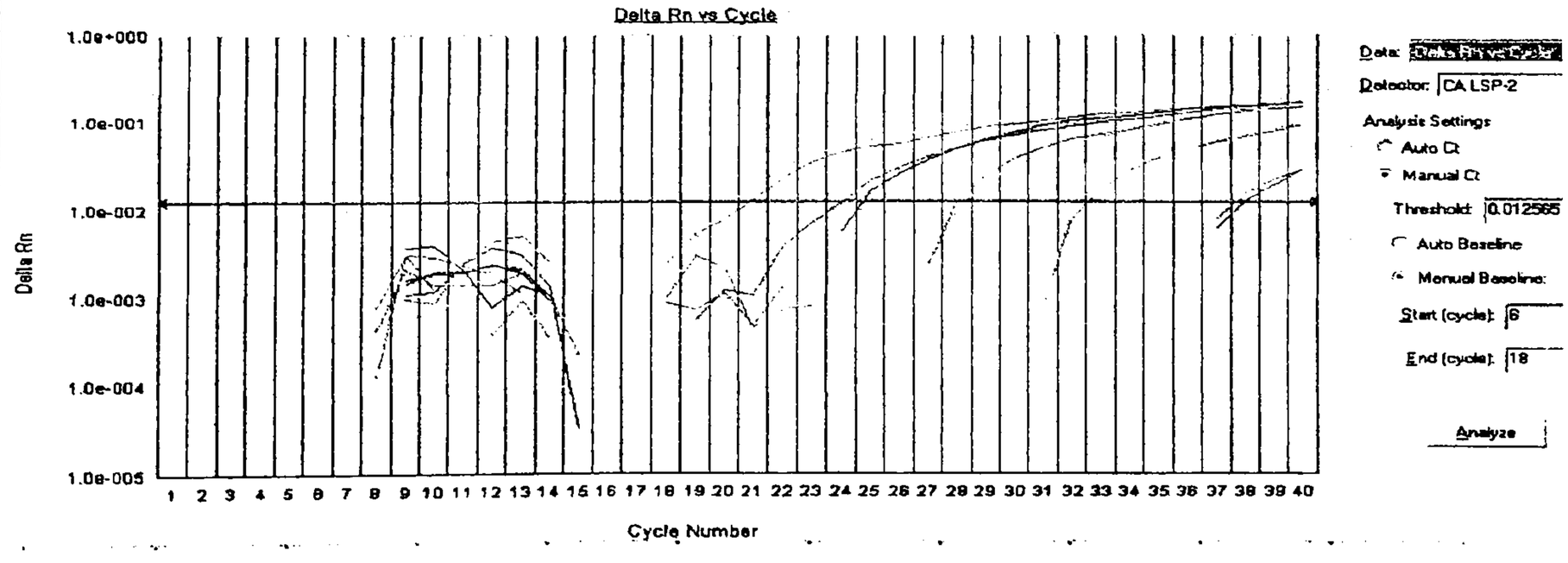
log view



LSP-2 - linear view



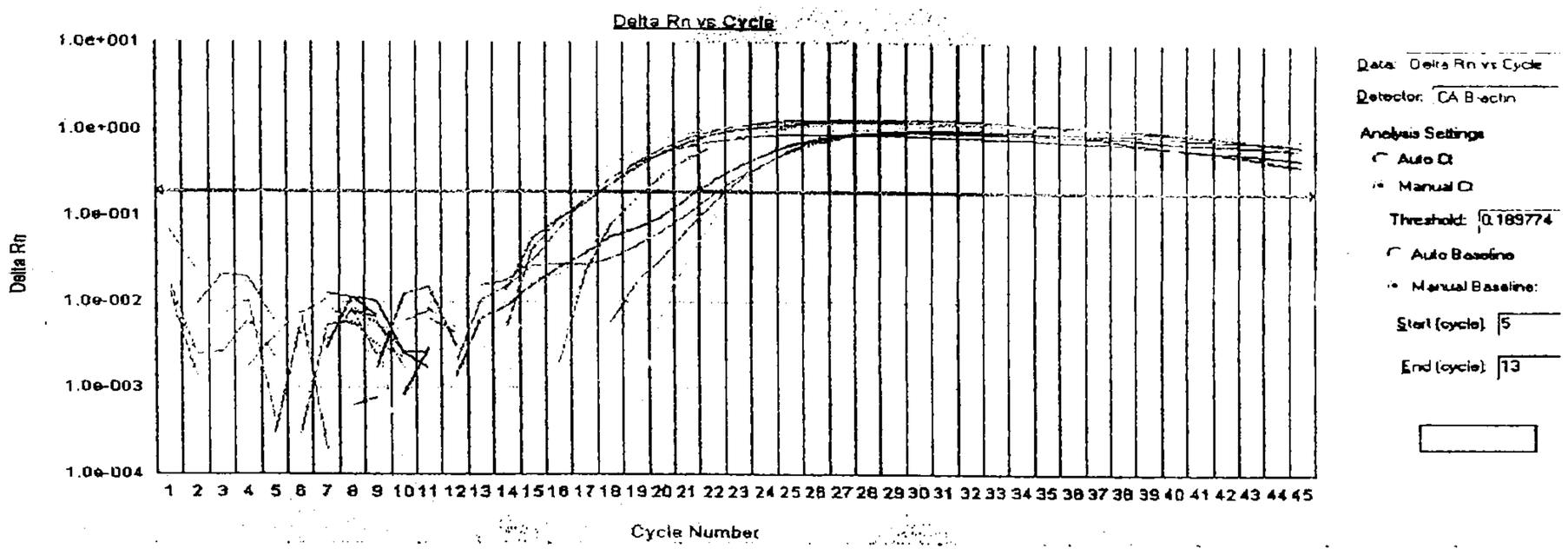
log view



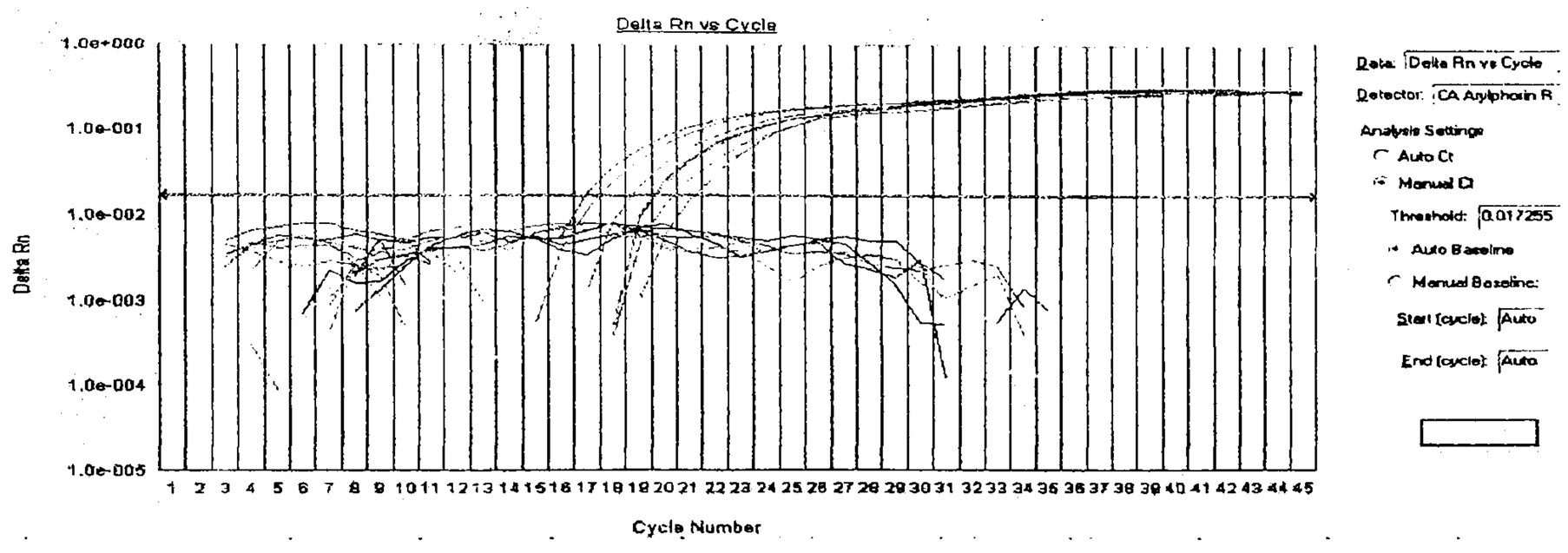
Appendix XI.

Example amplification plots for the first five pupal timepoints for the four gene regions.

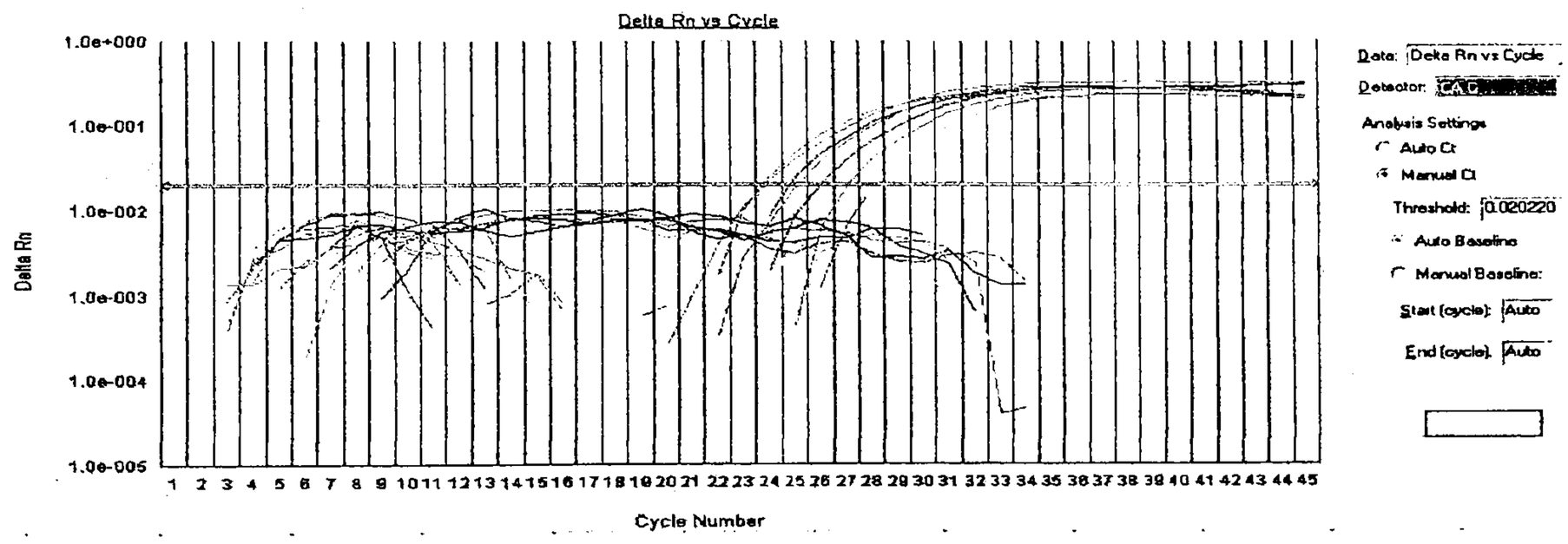
Actin



Arylphorin Receptor



Primer G



LSP-2

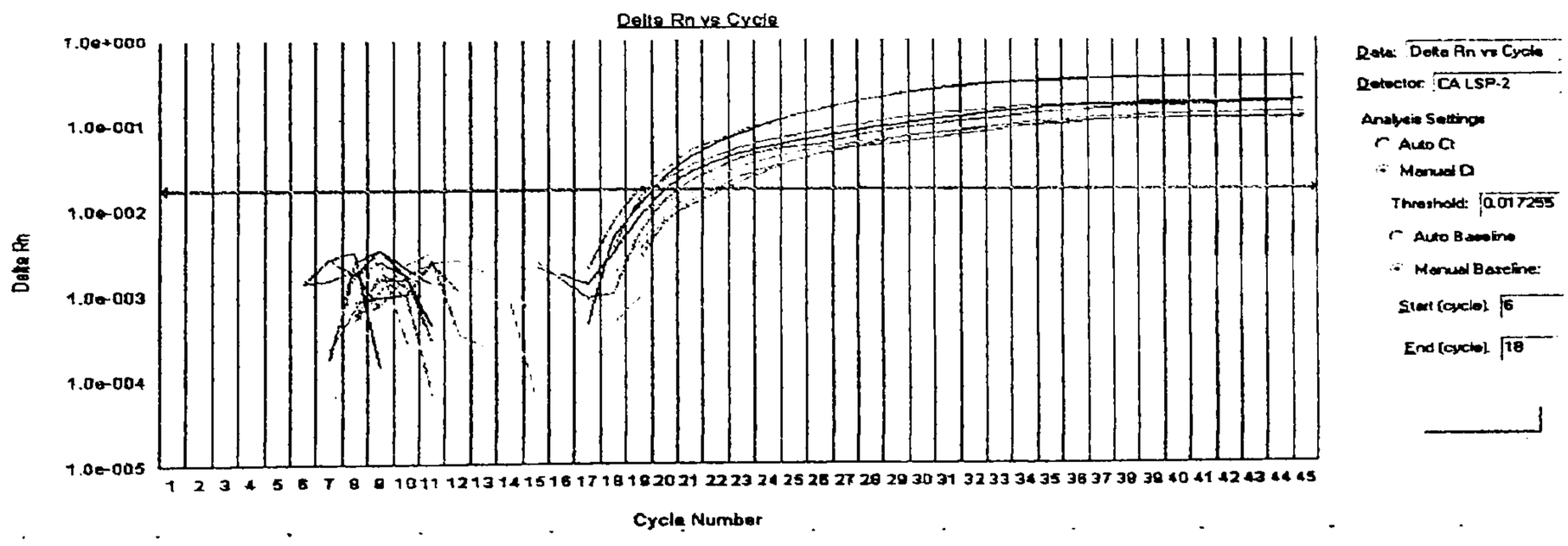


Table of raw CT values for each pupal timepoint for each primer/probe set.

ADH	Sample	Actin	Aryl R	Primer G	LSP-2
2880	1	17.29	21.14	23.69	21.87
	2	16.42	21.25	24.13	21.55
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
3360	1	17.56	19.49	24.73	19.46
	2	17.28	18.65	23.77	18.96
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
3840	1	19.21	19.58	25.1	19.49
	2	19.26	19.14	25.51	19.36
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
4320	1	21.37	16.89	26.03	20.03
	2	22.13	17.38	27.02	20.5
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
4800	1	22.43	20.3	28.14	21.24
	2	23.03	22.43	28.15	22.27
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
5760	1	23.06	21.43	28.33	21.66
	2	23.1	21.55	27.96	21.87
	NTC	35.73	Undetermined	Undetermined	Undetermined
6240	1	21.11	21.18	24.81	18.97
	2	20.88	20.68	25.31	19.42
	NTC	36.5	Undetermined	Undetermined	Undetermined
6720	1	21.22	21.81	26.41	19.28
	2	20.59	22.86	25.45	19.38
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
7200	1	28.82	31.29	32.59	30.05
	2	27.33	30.47	32.56	27.98
	NTC	39.79	Undetermined	Undetermined	Undetermined
7680	1	19.18	19.63	23.13	20.22
	2	19.51	19.74	23.08	20.13
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
8640	1	29.57	28.9	30.9	30.66
	2	28.22	27.49	30.04	29.28
	NTC	38.48	Undetermined	Undetermined	Undetermined
9120	1	19.77	22.31	19.42	23.74
	2	19.55	21.29	19.04	23.52
	NTC	40.5	Undetermined	Undetermined	Undetermined
9600	1	25.72	30.11	29.22	30.33
	2	25.34	31.26	28.4	32.36
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
10080	1	22.4	28.63	23.33	30.46
	2	22.62	28.54	23.68	31.69
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
10560	1	18.04	25.27	18.52	25.52
	2	17.92	22.22	18.25	24.28
	NTC	Undetermined	Undetermined	Undetermined	Undetermined
Plate 1 Negative	1	Undetermined	Undetermined	Undetermined	Undetermined
	2	Undetermined	Undetermined	Undetermined	Undetermined
Plate 2 Negative	1	39.42	Undetermined	Undetermined	Undetermined
	2	Undetermined	Undetermined	Undetermined	Undetermined
Plate 3 Negative	1	Undetermined	Undetermined	Undetermined	Undetermined
	2	36.25	Undetermined	Undetermined	44.26